# STUDIES ON THE ACQUISITION, EXPRESSION AND DISRUPTION OF MAMMALIAN SPERM MOTILITY

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## Contents

Except where acknowledgement is made by reference, the experiments described in this thesis were the unaided work of the author. All experiments were performed in the Medical Research Council's Unit of Reproductive Biology in Edinburgh.

D.R. White
October 1987

To Mum and Dad.

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## Preface

The studies detailed in this thesis were supported by a fellowship from Family Health International. This funding was obtained on the condition that at least a portion of the research would concentrate on applied aspects of fertility control in the male, with special reference to the actions of sulphasalazine, gossypol and propranalol, a diverse group of antifertility agents known to affect sperm motility. The results of these investigations have been detailed in section B of this thesis, entitled "Male Contraception".

In addition to these topics, a series of investigations into the fundamental mechanisms involved in the cellular control of sperm motility were performed. These studies are summarised in section A, "Fundamental Biology of Sperm Movement" .

## Abstract

Mammalian spermatozoa attain the ability to initiate progressive motility during their passage through the epididymis. In many species the movement characteristics of these cells are further modified in the female tract to produce a state of "hyperactivation", which is believed to facilitate penetration of the oocyte vestments, particularly the zona pellucida. The exact biochemical mechanisms involved in the development and expression of motility, and the way in which these processes are modified by potential contraceptive agents, such as sulphasalazine, gossypol and propranolol, remain unknown. Since published evidence implicates important roles for prostaglandins and the intracellular messengers cyclic 3',5' adenosine monophosphate (cAMP), calcium and pH in this context, this study investigated the involvement and interplay of these factors in the aquisition and expression of sperm movement.

In contradiction to previously published data in other species, cAMP levels were found to be higher in immotile caput epididymal spermatozoa, than in caudal epididymal spermatozoa, both in undiluted form and upon release into appropriate media. Both cell types expressed a rapid calcium-dependent, calmodulin-independent increase in cAMP content upon dilution to a concentration of 20 million spermatozoa/ml. This initial rise was abolished by dilution to one million/ml. Further investigations revealed that the rapid elevation of cAMP levels at the higher cell concentration was due to a factor present in epididymal plasma which may be a phosphodiesterase inhibitor. A more gradual increase in cAMP levels does occur in capacitating caudal spermatozoa incubated at this lower density, whilst caput levels remain low. This elevation precedes the appearance of hyperactivation, which, in turn, precedes the induction of the acrosome reacti .i.

Both hyperactivation and the rise in cAMP levels are abolished by incubation in medium with no added calcium (NAC). Neither phenomenon is restored by the addition of the phosphodiesterase inhibitor isobutyl methyl xanthine (IBMX), although similar treatment of caudal spermatozoa in medium MT-1 (containing 1.7mM calcium) increased cAMP levels, stimulated percentage motility and advanced the appearance of

hyperactivation. Addition of a membrane permeant analogue of cAMP to caudal spermatozoa in NAC also failed to evoke hyperactivation, whereas it mimicked the action of IBMX on caudal spermatozoa in MT-1

Incubation of caudal spermatozoa in MT-1 with the calmodulin antagonist calmidazolium slightly reduced cAMP levels and totally abolished coordinated sperm movement. Addition of IBMX in the presence of calmidazolium increased cAMP content to levels higher than those expressed in MT-1 alone, but did not restore coordinated motility.

To determine why caput spermatozoa failed to show an elevation of cAMP levels during prolonged incubation, as well as the mechanisms responsible for this increase in capacitating caudal spermatozoa, intracellular calcium concentration and pH were determined for both cell types. No overt difference in calcium content or internal pH exists between caput and caudal spermatozoa, with both cell types exhibiting a rise in calcium levels and a fall in pH over a time course of five hours. However, calcium levels declined significantly in caudal spermatozoa at the time when maximal levels of hyperactivation are being expressed.

In addition to these fundamental studies suggesting a role for cAMP in the expression of hyperactivation, the data published in this thesis also shed light on the mechanisms by which the contraceptive agents sulphasalazine, gossypol and propranolol influence sperm motility.

# Part A. Fundamental Biology of Sperm Movement

# Chapter 1. General Introduction

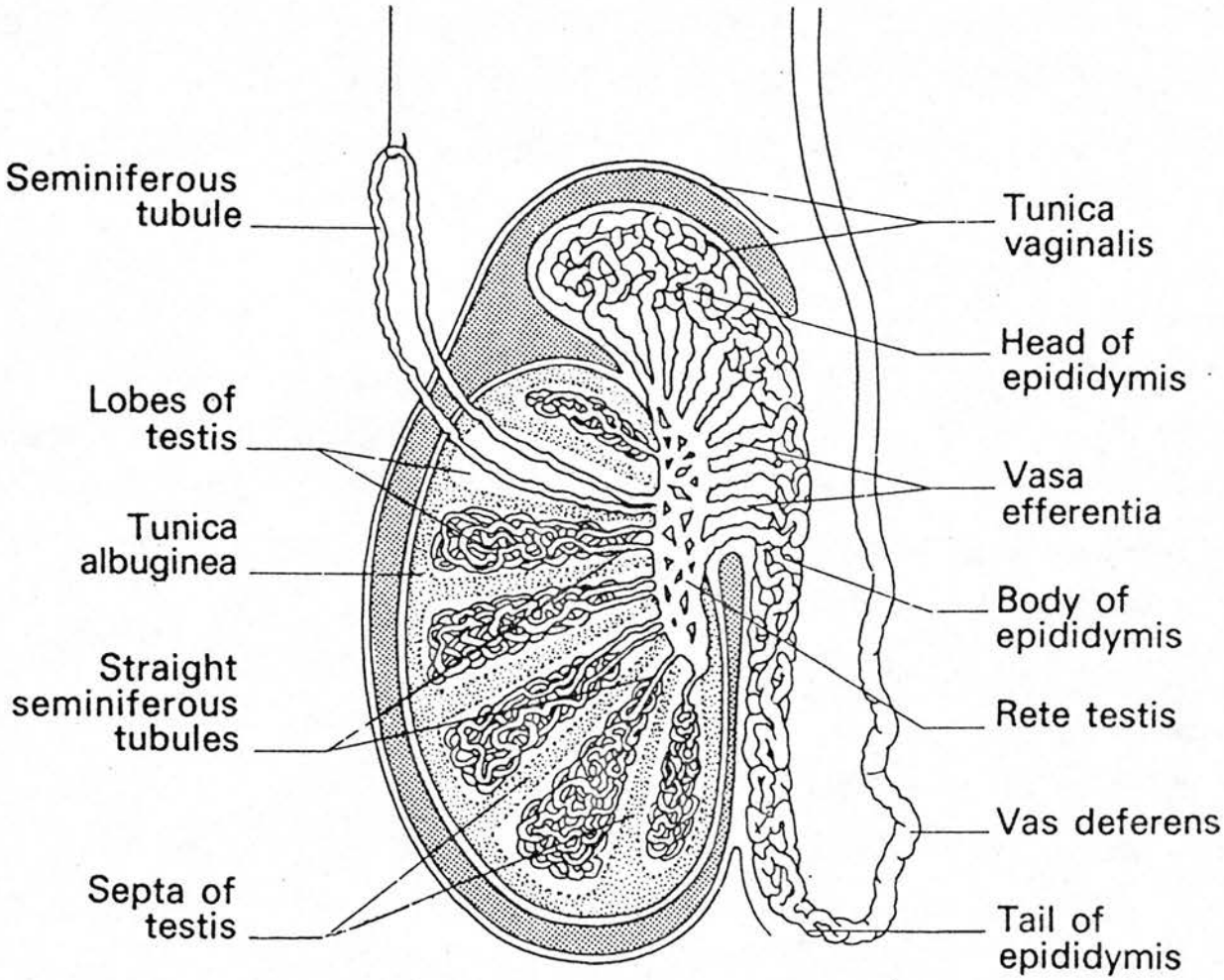



Figure 1. Section through the adult human testis showing the structure. (From Johnson M, Everitt B (eds): Essential Reproduction. Blackwell, London, 1984)

The purpose of this introduction is to acquaint the reader with the biological changes that spermatozoa undergo after they have completed their development in the testis, with special reference to alterations in the spermatozoa's capacity for movement. The changes which occur in the epididymis are detailed first, followed by an account of the alterations undergone by spermatozoa during their passage through the female genital tract. The significance of these changes is then discussed, and the biochemical mechanisms by which these alterations are thought to be mediated is introduced.

## A. Biology of the Epididymis

i. Background. The epididymis is a single duct which receives spermatozoa from the vasa efferentia and transports them distally to the vas deferens. Its origins lie in the mesonephric (Wolffian) duct; the so called "primitive embryonic kidney" (Gier and Marion, 1970). This organ has evolved in such a way that in mammals it has come to be utilised exclusively for reproductive purposes.

The epididymal duct is highly convoluted in shape, and is tightly packed within an extension of the testicular tunica albuginea to form the epididymis proper (Fig 1). On the basis of its shape, this organ is conventionally divided into three parts; the caput (head), which receives spermatozoa from the efferent ducts of the testis, the corpus (body), a narrow intermediate connection, and finally the cauda (tail), where spermatozoa are stored prior to ejaculation. These gross anatomical divisions are, however, misleading, as the exact regional boundaries are indistinct and vary from species to species (Glover and Nicander, 1971). In the rat and hamster for instance, the vasa efferentia occupies only the most proximal margin of the caput epididymis, whereas in man, a large portion of the caput region is not epididymal tissue at all, but is an extensive vasa efferentia (Baumgarten et al, 1971; Fig 1). Thus, information concerning a particular epididymal region from one species may not necessarily translate well to another.

The first recorded observations on the epididymis were made by Aristotle around 400BC (Thompson, 1910). This enterprising scholar provided the first clear and detailed description of testicular anatomy and function, and also produced suprisingly modern illustrations of the anatomy of the testis, efferent ducts, epididymis and vas deferens. Indeed, the term epididymis itself comes from the Greek, "didymi", meaning twins, which was used by this race to describe the paired testes.

With the advent of the light microscope, Johan Ham, a medical student at Leiden, brought a curious cell type in semen to the attention of the distinguished Dutch microscopist Antony van Leeuwenhoek. He subsequently established the existence of spermatozoa in the seminal fluid of a variety of species, and presented his observations to the Royal Society (Leeuwenhoek, 1679; Bodemer, 1973). The following year, an extensive study of the anatomy of the testis was published by de Graaf (Jocelyn and Setchell, 1972). By the nineteenth century, great advances had been made in the understanding of male fertility, and especially of testicular physiology. However, it appears that over this entire period the epididymis was regarded solely as a canal which facilitated the passage of spermatozoa from the testis to the penis. It was not until the very end of the nineteenth century that some function was ascribed to this organ, when it was noted that patterns of sperm motility changed within the epididymis (Hammar, 1897; Walker, 1899). Tournade (1913) confirmed these findings by showing that the capacity of spermatozoa for sustained progressive motility increased during their progression through the epididymis, and on the basis of this observation he proposed that spermatozoa must undergo a "ripening" process during their epididymal passage.

ii. Epididymal development of the capacity for fertilization. The first true evidence of functional maturation within the epididymis came from studies by Young (1931), who obtained pregnancy rates of 68% in oestrous guinea-pigs inseminated with spermatozoa collected from the distal part of

the cauda epididymis, in comparison to a figure of 33% obtained with equivalent samples from the proximal region. It has since been shown, using both in vivo and in vitro techniques, that spermatozoa from the rabbit (Nishikawa and Waide, 1952; Bedford, 1963a, 1966; Orgebin-Crist, 1967a; Paufler and Foote, 1968; Igboeli and Foote, 1969), rat (Blandau and Rumery, 1964; Dyson and Orgebin-Crist, 1973; Paz et al, 1978; Brackett et al, 1978), Syrian hamster (Horan and Bedford, 1972; Cummins, 1976; Gonzalez Echeverria et al, 1984), Chinese hamster (Dacheux and Paquignon, 1980; Yanagimachi, 1985), mouse (Pavlok, 1974; Hoppe, 1975), guinea pig (Young, 1931; Shilon et al, 1978; Williamson et al, 1980), boar (Hunter et al, 1976; Holtz and Smidt, 1976), ram (Fournier-Delpech, 1977, 1979; Voglmayr et al, 1978), bull (Amann and Griel, 1974), marmoset (Moore, 1981) and human (Hinrichsen and Blaquier, 1980; Schoysman, 1981; Moore et al, 1983) are infertile upon their exit from the testis, and only gain the ability to fertilize eggs during their passage through the epididymis. That the ability of the epididymis to support the maturation of spermatozoa depends upon the presence of androgens was shown by the loss of this capacity upon castration or hypophysectomy (Orgebin-Crist, 1973; Dyson and Orgebin-Crist, 1973; Orgebin-Crist and Davies, 1974; Lubicz-Nawrocki and Glover, 1974; Lubicz-Nawrocki, 1976). In vitro experiments have subsequently confirmed the androgen dependance of sperm maturation within the epididymis (Orgebin-Crist and Tichenor, 1973; Orgebin-Crist et al, 1976; Orgebin-Crist and Jahad, 1978).

iii. Epididymal contribution to sperm maturation. There has been much debate as to whether the maturation of spermatozoa within the epididymis is reliant upon the activity of some specific region of this organ, or whether spermatozoa possess an intrinsic capacity for maturation after spermiation, and thus utilise the epididymal duct merely as a favourable but nonspecific environment in which to develop their capacity for fertilization.

The consensus opinion amongst early workers was that the

epididymal epithelium must play a specific role in inducing changes in epididymal spermatozoa (Tournade 1913; Stigler, 1918; Benoit, 1921, 1926; Redenz, 1924, 1926; Von Lanz, 1926). However, from experiments where ligation of the caput epididymis produced an increase in the fertilization rate achieved by spermatozoa trapped within this region, it was postulated that the passage of time, rather than some specific property of epididymal secretion, determined the functional state of spermatozoa, and that once released from the Sertoli cell, the spermatozoon was committed to an ageing process, during which functional competence was achieved (Young 1929a,b, 1931; Young and Simeone, 1930; Simeone and Young, 1931; Simeone, 1933).

Subsequent studies appeared to provide support for this theory. In the rabbit, where spermatozoa normally acquire the capacity for fertilization in the distal corpus epididymis (Bedford, 1966), ligation of this region led to the appearance of some fertile spermatozoa amongst those held in the distal caput (Gaddum and Glover 1965; Bedford, 1967), or the proximal corpus (Orgebin-Crist, 1967a; Paufler and Foote, 1968). Furthermore, recipients of epididymovastostomies, a process by which epididymal blockage is overcome by connection of the caput to the vas deferens (Young, 1951), have been reported to subsequently father children (Young, 1970).

These studies would therefore appear to suggest that the corpus and caudal regions of the epididymis are not required for the induction of normal functional competence in spermatozoa from either the rabbit or human. However, occlusion of the epididymal duct has been shown to drastically alter the structure of the epididymal epithelium (Glover, 1962, 1969; Bedford, 1967; Orgebin-Crist, 1969). Thus, when the passage of spermatozoa through the epididymis is halted by obstruction, the whole character of the duct above the blockage becomes altered, and a completely abnormal situation ensues. Hence conditions in a blocked epididymis are not comparable to those prevailing in an intact organ. Subsequent studies on patients who have undergone epididymovastostomy have revealed that the likelihood of producing functionally competent spermatozoa is extremely low

if the vas deferens is anastomosed to the proximal 7-10mm of the epididymis (Silber, 1978; Schoysman, 1981). Furthermore, spermatozoa recovered from the corpus epididymis of fertile men fail to fertilise ova in vitro (Mahadevan and Trounson, 1985), and ligation of the epididymis does not promote the fertilizing ability of spermatozoa recovered from the testis (Cooper and Orgebin-Crist, 1975, 1977) or proximal caput epididymis (Gaddum and Glover, 1965; Bedford, 1967; Orgebin-Crist, 1967a, 1969; Cummins, 1976). The balance of evidence therefore indicates that the maturation of spermatozoa within the epididymis is dependent upon some component(s) of epididymal origin (Orgebin-Crist, 1969; Cummins and Orgebin-Crist, 1971; Orgebin-Crist et al, 1975; Cummins, 1976; Holtz and Smidt, 1976). This requirement cannot, however, be met simply by exposing immature spermatozoa to the secretory products of the distal portion of the epididymis (Cooper and Orgebin-Crist, 1975, 1977; Holtz and Smidt, 1976; Hunter et al, 1978; Voglmayr and Gandhi, 1978; Voglmayr et al, 1978; Peterson et al, 1984), and it is thus thought that sperm maturation involves a complex interaction between the maturing spermatozoa and the luminal fluid and epithelium of the epididymis (Orgebin-Crist and Jahad, 1978).

iv. Epididymal development of the capacity for movement. The acquisition of fertilizing ability within the epididymis runs parallel to an increased potential for progressive movement, suggesting that a relationship exists between these two factors (Dacheux and Paguignon, 1980). It is known that spermatozoa from the rat (Blandau and Rumery, 1964; Gaddum, 1968; Fray et al, 1972; Burgos and Tovar, 1974; Wyker and Howards, 1977; Morton et al, 1978; Hinton et al, 1979a; Turner and Giles, 1981), rabbit (Orgebin-Crist, 1967a, 1969; Gaddum, 1968; Acott et al, 1979; Pholpramool and Chaturapanich, 1979), hamster (Morton et al, 1978; Kann and Serres, 1980), mouse (Morton et al, 1978), ram (Fournier-Delpech et al, 1977; Amann et al, 1982; Dacheux et al, 1983), boar (Acott et al, 1979; Dacheux et al, 1983), bull (Cascieri et al, 1976; Morton et al, 1978; Acott et al, 1983; Carr

and Acott, 1984), guinea pig (Shilon et al, 1978), cat, dog, monkey, bear, elephant (Acott et al, 1979) and human (Belonoschkin, 1942; Mooney et al, 1972; Bedford et al, 1973, Morton et al, 1978) all acquire the potential for movement during their passage through the epididymis, and it is assumed that this phenomena occurs in every mammalian species (Bedford, 1975; Hamilton, 1975; Orgebin-Crist, 1975; Bedford, 1979).

Although spermatozoa isolated from the seminiferous tubules of mammals are completely immotile (Hinton et al, 1979a), caput epididymal spermatozoa from rabbits (Orgebin-Crist, 1967a; Gaddum, 1968; Acott et al, 1979; Pholpramool and Chaturapanich, 1979), rats (Blandau and Rumery, 1964; Fray et al, 1972; Wyker and Howards, 1977; Hinton et al, 1979a; Turner and Giles, 1981) and hamsters (Kann and Serres, 1980) all exhibit a tendency to move in circles. In contrast, spermatozoa from the human caput epididymis exhibit only weak vibrations, and never express such circular movements (Mooney et al, 1972; Bedford et al, 1973). However, as the fluid environment within the lumen of the caput epididymis has a high osmolarity (Levine and Marsh, 1971; Johnson and Howards, 1976; D'Addario et al, 1980) the movement patterns expressed by immature spermatozoa may reflect artificially induced changes in the flagellum resulting from incubation in culture medium which is hypo-osmotic with respect to their normal milieu (Serres and Kann, 1984). In the rat, hamster and mouse, the movement of immature cells is associated with an apparent "stiffness" of the mid-piece, preventing the degree of rotation required for mature patterns of motility (Fray, 1972; Kann and Serres, 1980; Saling, 1982). This contrasts with the situation in bovine caput spermatozoa, in which the mid-piece appears to be too flexible, resulting in the rotation rate in these cells occuring at a lesser rate than in mature spermatozoa (Acott and Hoskins, 1983; Acott et al, 1983).

v. Epididymal contribution to sperm movement. Investigations of the involvement of the epididymis in the maturation of sperm motility have relied

heavily upon the use of ligation techniques, which have been shown to drastically alter the nature of the duct proximal to the site of ligation (Glover, 1962, 1969; Bedford, 1967; Orgebin-Crist, 1969). Although in vitro ageing of ram and bull spermatozoa in rete testis (Voglmayr et al, 1967; Voglmayr et al, 1970), or epididymal (Voglmayr et al, 1977) fluid has no stimulatory effects upon any aspect of their motility, such studies have shown that spermatozoa trapped by these means in the seminiferous tubules (Glover, 1962; Orgebin-Crist, 1967b), rete testis (O'Shea and Voglmayr, 1970; Cooper and Orgebin-Crist, 1975, 1977), caput (Glover, 1962; Gaddum and Glover, 1965; Bedford, 1967; Orgebin-Crist, 1967b; Gaddum, 1968; Burgos and Tovar, 1974) or corpus (Horan and Bedford, 1972; Cummins, 1976) epididymides do show an increase in flagellar beat rate. However, spermatozoa treated in this manner never acquire the capacity for forward motility, suggesting that this ability is conferred upon spermatozoa by some component of epididymal origin, and is not simply due to an ageing process. This theory is supported by castration and androgen replacement studies, which alternately switch off and re-initiate epididymal function, respectively preventing and maintaining the ability of the epididymis to induce forward motility in spermatozoa trapped in the corpus region (Orgebin-Crist, 1973).

Lindholmer (1974) observed that human seminal plasma could induce forward motility in caput epididymal spermatozoa recovered from patients suffering from obstructive azoospermia. Subsequently it was shown that progressive motility could be induced in immature spermatozoa from the bull by incubation in the presence of bovine seminal plasma following activation of the cells with phosphodiesterase inhibitors (Hoskins et al, 1975b; see below). This ability was subsequently shown to be due to a 37500 Dalton glycoprotein named forward motility protein (FMP) (Acott and Hoskins, 1978; Hoskins et al, 1978), of epididymal origin (Brandt et al, 1978), which binds tightly to spermatozoa prior to their entrance into the proximal portion of the cauda epididymis (Acott and Hoskins, 1981). Similar effects have been attributed to a factor in the caudal fluid of the hamster (Kann and Serres, 1980; Serres and Kann, 1984), although forward

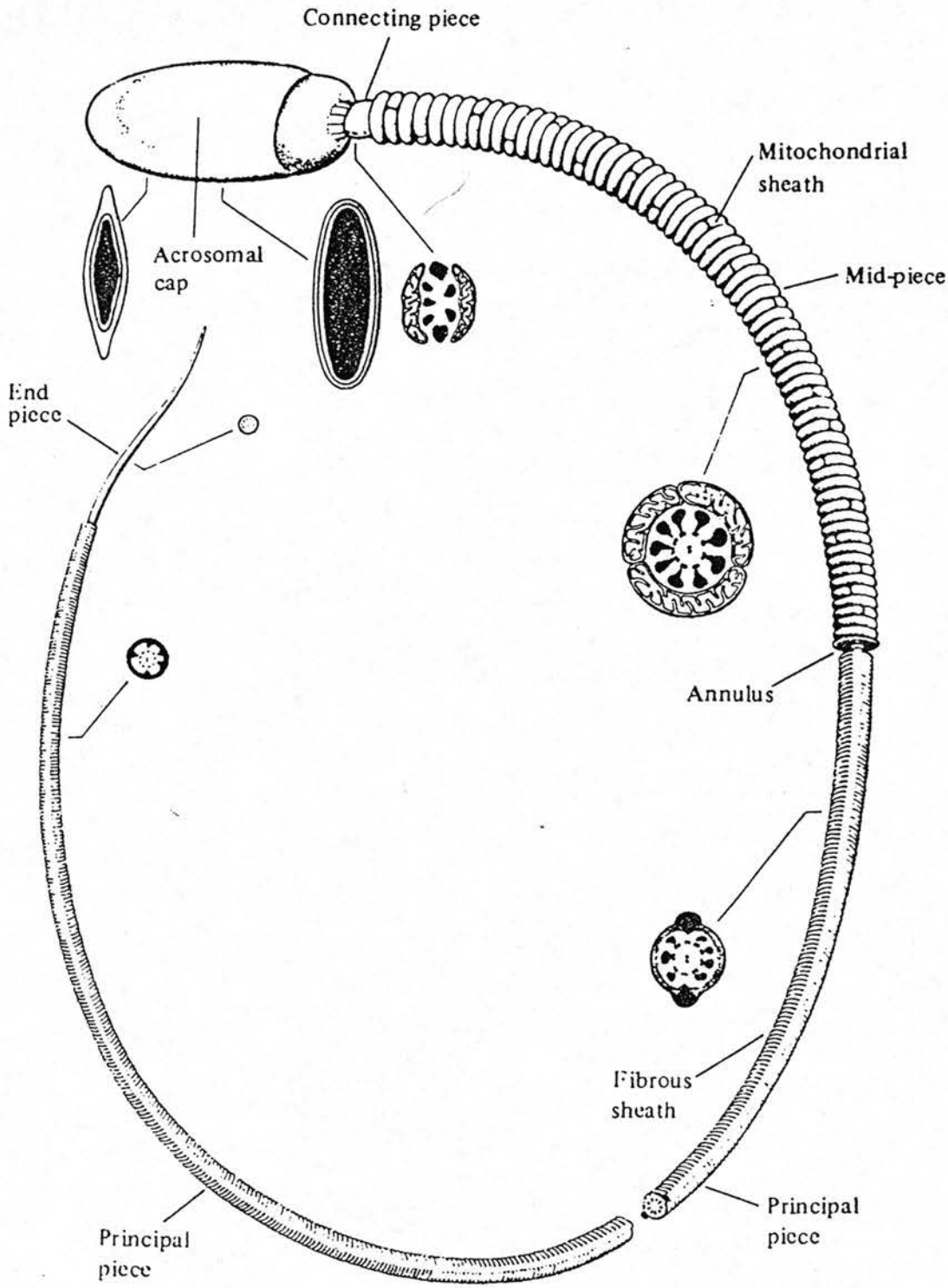



Figure 2. Diagram of a primate spermatozoon with the cell membrane removed to show the internal structure. (From D W Fawcett. Devel. Biol 44: 394 (1975))

progression in immotile spermatozoa from the ram (Amann et al, 1982), boar (Dacheux and Paquignon, 1980) and rat (Turner and Giles, 1982) cannot be induced by caudal plasma.

The binding of epididymal glycoproteins to the surface of developing spermatozoa had previously been observed (Koehler, 1973; Gordon et al, 1975; Nicolson and Yanagimachi, 1974; Nicolson et al, 1977), and because of the acidic properties of such molecules, such a process would account for the increasingly negative charge found on the sperm tail during epididymal transit (Bedford, 1963b; Cooper and Bedford, 1971; Yanagimachi et al, 1972). The ability of this FMP to increase beat frequency and power output is not species specific (Acott et al, 1979), and although it has been shown that part of this protein's action was due to a reduction of the binding of sperm heads to the glass surfaces in the motility assessment chamber (Stephens et al, 1981), this factor appears to act by reducing the flexibility of the flagellar mid-piece (Acott and Hoskins, 1983; Acott et al, 1983; Serres and Kann, 1984).

In the hamster, anti-calmodulin drugs have been shown to mimic the effects of FMP upon immature spermatozoa, and it has thus been proposed that this protein's action may be mediated via this calcium-binding peptide (Serres and Kann, 1984). However, calmodulin inhibitors are known to have completely opposite effects upon ejaculated dog spermatozoa, converting mature motility patterns into movements reminiscent of those displayed by caput spermatozoa (Tash and Means, 1982).

vi. Morphological changes in sperm during epididymal transit.. The mammalian spermatozoa is a small, elaborate, highly specialised and condensed cell consisting of two major parts; the head and the tail (Fig 2). The principal component of the head is the nucleus, which is covered anteriorly by a characteristic cap-like structure, the acrosome, and posteriorly by a cytoplasmic sheath, the post-nuclear region. The tail is a long flagellum that is differentiated into four regions; the neck or connecting piece, the mid-piece, which contains the mitochondrial sheath, the principal

piece and the end piece.

Ultrastructural modifications of spermatozoa are known to accompany epididymal transit. Of these, the most noticeable change is the migration of the cytoplasmic droplet, which gradually moves distally along the flagellum, and is usually shed from the tail midpiece (Redenz, 1926). This droplet represents a cytoplasmic remnant of the residual body from which the spermatid was separated during spermiation (Fawcett and Phillips, 1969). The role of this protoplasmic droplet is unknown. However, it has been demonstrated that these droplets have close biochemical and strutural similarities to lysosomes (Dott and Dingle, 1968; Garbers et al, 1970; Moniem and Glover, 1972; Harrison and White, 1972), and it may be that this structure interacts with the sperm membrane surrounding the mitochondria, thus affecting the activity of this organelle.

The morphology of the acrosome also changes during epididymal maturation. Originally reported as a shrinkage of the entire sperm head (Mukherjee and Bhattacharya, 1949), this has now been shown to be caused by a reduction in the size of the acrosome (Fawcett and Phillips, 1969; Jones, 1971; Bedford and Nicander, 1971; Bedford, 1963, 1965). These changes are perhaps most dramatically illustrated in the guinea pig, where the acrosome of testicular spermatozoa is flattened and large, whilst in mature spermatozoa, it assumes a more rounded and smaller appearance (Fawcett and Hollenberg, 1963).

However, the ultra-structure of the sperm tail does not appear to change during epididymal maturation (Olson and Sammons, 1980), despite the observation that the total disulphide bond content of the spermatozoan tail increases concomitant with this process (Calvin and Bedford, 1971; Calvin et al, 1973; Bedford and Calvin, 1974). Using demembranated sperm models, in which flagellar beating can be re-initiated by treatment with ATP (Bishop and Hoffman-Berling, 1959; Morton, 1973; Lindemann and Gibbons, 1975; Lindemann, 1978; Lindemann et al, 1980), it has been shown that immature spermatozoa are capable of exhibiting coordinated flagellar movement (Mohri and Yanagimachi, 1980; Treetipasatit and Chulavatnatol,

1982; Yeung 1984, 1986; White and Voglmayr, 1986). Thus, although the flagellar system of spermatozoa is thought to undergo some modification during epididymal maturation (Mohri and Yanagimachi, 1980; Yeung 1984, 1986; White and Voglmayr, 1986), it would appear that the motor apparatus of these cells is already functionally assembled upon their exit from the testis.

vii. Epididymal storage of spermatozoa. Spermatozoa stored in the cauda epididymis possess the full potential to express motility upon dilution. However, as early as 1925 it was reported that mature guinea pig spermatozoa remained immotile whilst still in their native fluid (Cody, 1925). It is now known that spermatozoa are immotile whilst resident in the cauda epididymis of rats (Jones, 1978; Morton et al, 1978; Turner and Howards, 1978; Usselman and Cone, 1983; Turner and Reich, 1985; Carr et al, 1985), hamsters (Jones, 1978; Morton et al, 1978; Turner and Reich, 1985; Carr et al, 1985), mice (Morton et al, 1978), guinea pigs (Turner and Reich, 1985; Carr et al, 1985) and man (Turner and Reich, 1985). It is commonly assumed that the storage of spermatozoa in a quiescent state during their sojourn in the cauda epididymis allows energy stores to be conserved for the journey through the female genital tract and for the fertilization process (Mann and Lutwak-Mann, 1981; Zanaveld and Chatterton, 1982). However, spermatozoa from rams (Jones, 1978), rabbits (Jones, 1978; Morton et al, 1978; Usselman and Cone, 1983; Turner and Reich, 1985) and rhesus monkeys (Usselman and Cone, 1983) are motile in undiluted fluid from the cauda epididymis. Bull spermatozoa also exhibit flagellar movement prior to ejaculation (Cascieri et al, 1976; Morton et al, 1978; Carr and Acott, 1984; Pholpramool et al, 1985). Although the extent of sperm movement within the cauda epididymis evidently varies considerably between studies, it never equals the levels expressed upon release into media. An explanation for these differing strategies awaits elucidation

## B. Post ejaculatory modifications to spermatozoa.

i. Capacitation. Spermatozoa which have completed epididymal maturation are still not competent to achieve fertilization immediately upon ejaculation. CR Austin (1951) first reported that eggs recovered from rabbits artificially inseminated prior to ovulation were mostly fertilized, whilst eggs from females inseminated after ovulation rarely showed signs of penetration. Although in the light of subsequent studies this may be interpreted as a modification of the state of the oocyte, in view of this and a similar observation (Chang, 1951), it was proposed that spermatozoa required a period of maturation within the female genital tract before their potential for fertilization can be realised, and the term "capacitation" was coined to describe this phenomenon (Austin, 1952).

As spermatozoa recovered from the site of fertilization often lacked, or at least possessed highly modified acrosomes (Austin and Bishop, 1958), it was generally accepted that the process of capacitation included the loss of the acrosome during the exocytotic acrosome reaction. However, following the observation that capacitated spermatozoa could be "decapacitated" (Chang, 1957; Bedford and Chang, 1962), it is now accepted that capacitation includes acquisition of the ability to acrosome react and to subsequently generate an altered area of plasma membrane around the equatorial segment capable of fusing with the oolemma (Bedford, 1970; Yanagimachi and Usui, 1974; Saling et al, 1978; Bedford, 1983), but does not include the actual undertaking of these physiological changes (Bedford, 1970).

ii. Hyperactivated motility. In the 1960's, spermatozoa were shown to exhibit a progressive increase in oxygen uptake and glycolytic activity during capacitation (Hamner and Williams, 1963; Mounib and Chang, 1964; Murdoch and White, 1967; Black et al, 1968; Iritani et al, 1969). It was subsequently shown that the nature of the flagellar beat pattern also

changed during the terminal stages of capacitation, accounting for the increase in metabolic activity. These altered movement characteristics were originally described for the hamster (Yanagamachi, 1969, 1970), and subsequently shown to occur in the guinea pig (Yanagimachi, 1972; Barros et al, 1973; Yanagamachi and Usui, 1974), dog (Mahi and Yanagamachi, 1976), mouse (Fraser, 1977), rabbit (Cooper et al, 1979; Suarez et al, 1983), bat (Lambert, 1981), marmoset (Moore, 1981), bottlenosed dolphin (Fleming, 1982), ram (Shams-Borhan and Harrison, 1981; Cummins, 1982), rhesus monkey (Boatman and Bavister, 1984) and human (Gould et al, 1983; Burkman, 1984). Although originally termed activation (Yanagimachi, 1970), this beat pattern has since been renamed hyperactivation (Yanagimachi, 1981) to avoid confusion with the initiation of motilty from quiescence which occurs at ejaculation in many species (see above).

Hyperactivated motility is not an artifact generated in vitro, as similar patterns of movement have been observed in spermatozoa both in situ and upon recovery from the female tract after mating (Yanagimachi, 1970; Fraser, 1977; Cooper et al, 1979; Katz and Yanagimachi, 1980; Overstreet et al, 1980; Cummins, 1982) The movement characteristics of spermatozoa exhibiting hyperactivated motilty differ dramatically from those of freshly ejaculated cells. Whereas recently ejaculated spermatozoa propagate three dimensional beats, and as a result swim in linear trajectories (Yeung and Wooley, 1984), hyperactivating spermatozoa generate vigorous, large amplitude planar beats (Yanagimachi, 1970; Yanagimachi and Usui, 1974; Fraser, 1977; Cooper and Woolley, 1982; Ishijima and Mohri, 1985), which are assymetrical, possibly as a result of the significantly one sided nature of the flagellar bend (Katz et al, 1978). As a result, hyperactivating spermatozoa move in a non-progressive, circular manner (Suarez et al, 1983), with the sperm heads tracing erratic trajectories (Katz and Yanagimachi, 1980).

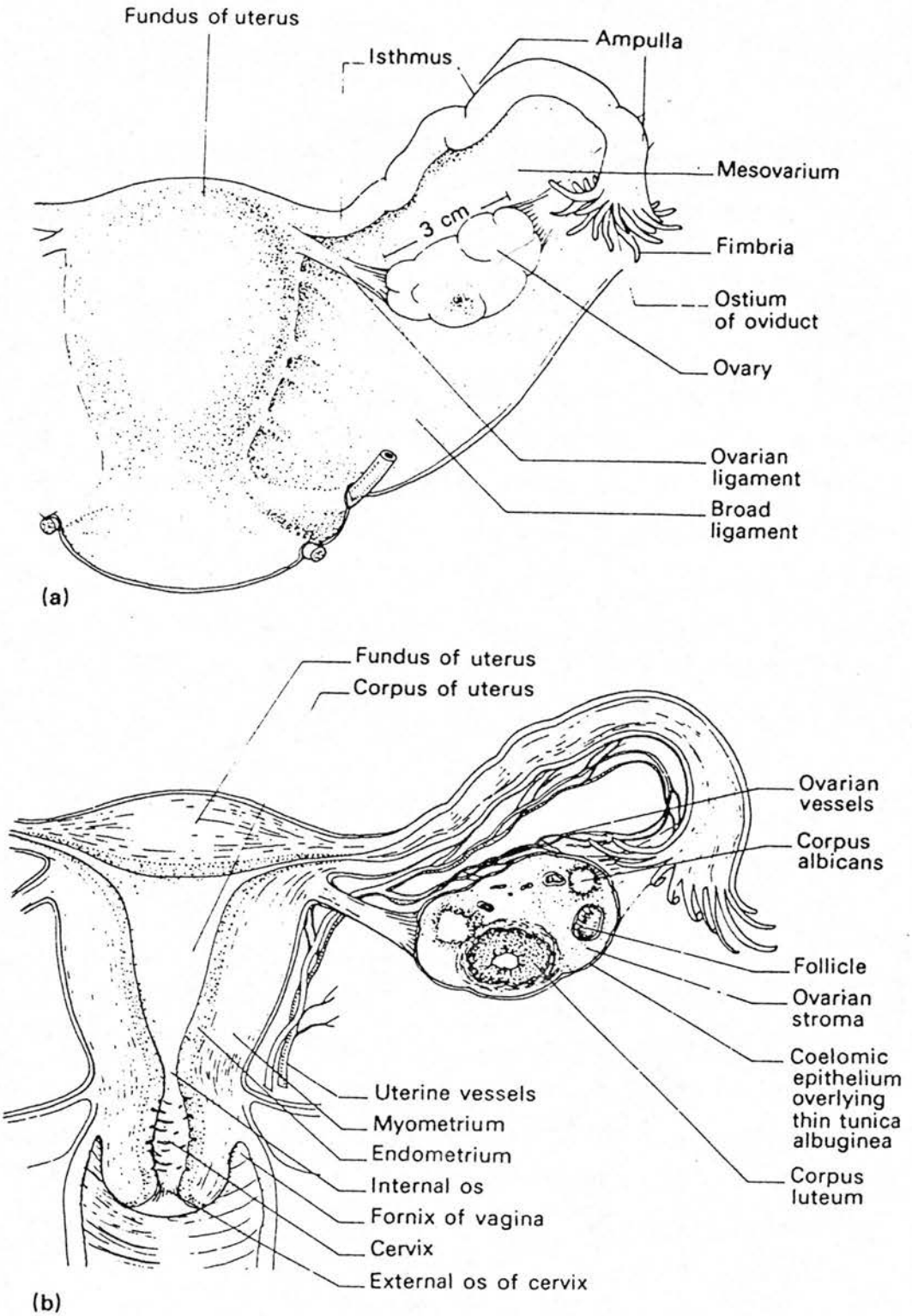



Figure 3. Posterior views of the human uterus and one oviduct: (a) intact; (b) sectioned. (From Johnson M, Everitt B (eds): Essential Reproduction. Blackwell, London, 1984)

## C. Requirement for motility.

What role does motility occupy in the fertilization process? Although early workers believed that spermatozoa propelled themselves through the epididymis (Redenz, 1926; Von Lanz, 1929), it is now clear that these cells contribute little to their own passage. The requirement for motility is confined to the spermatozoon's journey through the female tract (Fig 3 a, b). This process is initiated by the deposition of semen at ejaculation. In the rabbit, cow, sheep, goat and primate, the insemination site is intra-vaginal. Thus, in these species, the cervix presents the first barrier to sperm transport in the female (Mortimer, 1978). This is the case particularly in ruminants and primates, where copious quantities of cervical mucus exist (Katz and Overstreet, 1980). Passage of spermatozoa through this mucus appears to depend entirely upon their capacity for movement (Mortimer, 1978, 1983; Hunter 1980; Aitken et al, 1985, 1986) even in the rabbit (Noyes et al, 1959), where only small amounts of cervical mucus are found (Katz and Overstreet, 1980). However, the subsequent progress of spermatozoa into and through the uterus probably depends more upon myometrial contraction than upon sperm motility (Mortimer, 1978).

In the pig, dog, horse and rodent, the passage of semen through the cervix is achieved at ejaculation (Blandau, 1945), and spermatozoa are deposited almost directly into the uterus (Bedford, 1971; Mortimer, 1978). In these species, the uterotubual junction constitutes the major barrier to sperm transport through the female tract (Gaddum-Rosse, 1981), as passage through the uterus itself is facilitated by the thorough mixing of semen and uterine contents which follows mating in these species (Yamanaka and Soderwall, 1960). As immotile spematozoa from rams, rabbits and hamsters are capable of traversing the uterotubal junction (Setchell et al, 1969; Lambiase and Amann, 1973; Cooper and Orgebin-Crist, 1975; Cummins, 1976; Voglmayr et al, 1978), it is thought that in these species the passage of spermatozoa through this region is facilitated by movements of the female tract (Hunter et al, 1983). However as immotile spermatozoa appear to be

less efficient in completing this journey than fully motile cells (Orgebin-Crist, 1967a, 1968; Baker and Degen, 1972; Mortimer, 1977), a requirement for sperm motility must exist. The failure of non-motile spermatozoa from bulls and rats to enter the oviduct (Blandau and Rumery, 1964; Amann and Griel, 1974; Gaddum-Rosse, 1981) suggests that in these species, sperm transport into this area relies more heavily upon self propulsion.

The average length of time required for capacitation in the majority of species appears to lie somewhere between three and four hours (Hunter, 1987). However, the time span from mating at oestrus to the onset of ovulation can be up to forty hours (Hunter and Dziuk, 1968; Hunter, 1980). Thus, spermatozoa acummulate in the isthmus of the oviduct far in advance of the arrival of the oocyte in the ampulla (Harper, 1973; Hunter et al, 1980; Cummins and Yanagimachi, 1982). In order to maximise the efficiency of fertilization, it appears that the distal portion of the oviducal isthmus has evolved as a reservoir for spermatozoa (Hunter and Nichol, 1983). Spermatozoa are stored in this area in an arrested, pre-capacitated state during the pre-ovulatory interval (Hunter and Nichol, 1983; Hunter, 1984). Spermatozoa resident in the isthmus are practically immotile (Overstreet and Cooper, 1975; Cooper et al, 1979; Johnson et al, 1981; Suarez, 1987), although motility can be re-established by dilution and incubation in media or ampullary fluid (Overstreet et al, 1980; Johnson et al, 1981).

The ascent of spermatozoa to the ampulla appears to occur in two phases; the first, rapid-transport phase follows immediately after coitus, with spermatozoa being passively transfered to this site within minutes of insemination. However, such spermatozoa are non-motile, and pass into the peritoneal cavity prior to ovulation (Overstreet and Cooper, 1978). The second phase appears to be coordinated so as to coincide with the onset of ovulation (Harper, 1973a,b; Yanagimachi and Mahi, 1976; Shalgi and Kraicer, 1978; Overstreet et al, 1978; Overstreet and Cooper, 1979; Cummins and Yanagimachi, 1982). Although isthmic spermatozoa rarely express hyperactivation (Cooper et al, 1979; Cummins, 1982), a large percentage of spermatozoa exhibit hyperactivation in the ampulla

(Cummins, 1982; Cummins and Yanagimachi, 1982; Suarez et al, 1983). Spermatozoa may require to display this pattern of movement in order to progress from the isthmus to the ampulla of the oviduct (Overstreet and Cooper, 1979; Cummins, 1982; Cummins and Yanagimachi, 1982). However, the means by which sperm ascent is synchronised with ovulation is thought to depend upon passive aid from oviductal contractions (Battalia and Yanagimachi, 1979), which change in amplitude and frequency at ovulation (Salomy and Harper, 1971; Talo, 1974; Spilman, 1974). These, in turn, are probably regulated by the changes in ovarian steroid secretion which precede ovulation (Waterston and Mills, 1976).

The number of spermatozoa which actually reach the ampulla is very low, with a sperm/egg ratio of approximately unity appearing normal (Zamboni, 1972; Thibault, 1973; Shalgi and Kraicer, 1978; Cummins and Yanagimachi, 1982). It would thus appear that spermatozoa are much more efficient at fertilizing eggs in vivo than in vitro, where the number of sperm required to fertilize eggs is considerably higher (Fraser and Drury, 1975; Tsunoda and Chang, 1975; Siddiquey and Cohen, 1982). The reason for this difference is thought to be due to the existence of a positive selection procedure which operates upon spermatozoa as they pass through the various barriers of the female tract (Krzanowska, 1974; Mortimer, 1977).

The expression of hyperactivation is closely correlated with fertilizing potential (Fraser, 1981; Fleming and Yanagimachi, 1982). Although this pattern of movement may incidentally allow spermatozoa to resist confinement within mucosal folds (Suarez et al, 1983; Suarez and Osman, 1987), aid sperm transport to the ampulla (see above), and maximise the chance for contact between spermatozoa and the egg mass (Katz et al, 1978), it is thought that the primary function of hyperactivated motility is to increase the thrust generated by the flagellar beat (Katz et al, 1978; Dresdner and Katz, 1981; Johnson et al, 1981), facilitating the passage of spermatozoa through both the cumulus oophorus and the zona pellucida (Katz et al, 1986).

## D. Mechanisms by which spermatozoa acquire the capacity for movement

As detailed above, during their normal life cycle, mammalian spermatozoa may experience up to four transitional states with respect to their capacity for movement; The acquisition during epididymal maturation of the potential for coordinated, progressive movement, the expression of this ability upon ejaculation in those species in which epididymal spermatozoa are quiescent, the suppression of this motility during storage in the isthmus, and the expression of hyperactivated motility in the female genital tract. Elucidation of the mechanisms involved in these transformations, apart from providing the answers to fundamental biological questions, would be an important achievement for two diametrically opposed reasons. Firstly, this information would provide insights into naturally occuring asthenozoospermia, allowing the development of rational forms of treatment for such cases of male infertility. Secondly, knowledge of the mechanisms by which spermatozoa acquire the capacity for movement should be of strategic importance in the development of male contraceptive agents (Prasad et al, 1973; Hinton 1980; de Kretser, 1980; Fraser, 1982).

i. Energy metabolism in the epididymis. Sperm movement is generated by the active sliding of the axonemal doublets, which are situated longitudinally throughout the flagellum (Afzelius, 1959; Satir, 1968, 1974, 1979). The motility of demembranated spermatozoa depends upon the presence of ATP in the reactivation media (Morton, 1973; Rikmenspoel et al, 1978; Mohri and Yanagimachi, 1980; Yeung 1984, 1986), and this movement can be inhibited by selectively blocking dynein ATPase activity (Kobayashi et al, 1978; Bouchard et al, 1981), implying that the power for flagellar motion comes from the hydrolysis of adenosine triphosphate by ATPases on the dynein arms (Gibbons and Rowe, 1965). The ATP required for ATPase activity can be generated by both anaerobic glycolysis, utilizing

either glucose or fructose as a substrate, or respiration coupled with oxidative phosphorylation in the mitochondria (Rikmenspoel 1965; Rikmenspoel et al, 1969; Mann, 1983). However, different species place emphasis on different processes. For instance, glycolysis provides the major energy source for human sperm motility (Peterson and Freund, 1968; Hong et al, 1983), whereas motility in hamster and bull spermatozoa appears to depend primarily upon respiration (Fischer-Fischbein et al, 1985; Inskeep and Hammerstedt, 1985).

The rates of both glycolysis and respiration are lower in testicular than in ejaculated spermatozoa from the boar and ram (Dacheux et al, 1979; Dacheux and Paquignon, 1980; Hammerstedt, 1981; Inskeep and Hammerstedt, 1982), as is glucose consumption in guinea pig and bovine spermatozoa (Frenkel et al, 1973a, b; Hoskins et al, 1975a). However, the consumption of this substrate by rat spermatozoa does not appear to change during epididymal maturation (Paz et al, 1978). Levels of glyceraldehyde 3-phosphate dehydrogenase (GPD), which may be the rate limiting enzyme in sperm glycolysis (Hoskins et al, 1971; Hoskins et al, 1975a), increase in guinea pig and mouse spermatozoa as they pass from the caput to the corpus epididymis (Ford and Harrison, 1983), parallelling the acquisition of the potential for fertility and motility (Shilon et al, 1978; Williamson et al, 1980). In contrast, GPD activity in rat and hamster spermatozoa has been shown to decrease during epididymal maturation (Ford and Harrison, 1983). However, this enzyme may not play such an important role in these species, as glycolytic flux remains low when spermatozoa from these animals are incubated under aerobic conditions (Ford et al, 1979). Measurements of the other glycolytic enzymes have failed to reveal any clear patterns of change accompanying epididymal maturation (Terner et al, 1975; Hammerstedt 1975; Mongkolsirikieat and Boonsaeng, 1980).

Of all the possible energy substrates for epididymal spermatozoa, probably the most intensively studied has been carnitine. Increases in both the luminal fluid (Brooks et al, 1973, 1974; Hinton et al, 1979b; Casillas et al, 1984; Besançon et al, 1985) and spermatozoal (Casillas, 1973; Casillas and

Chaipayungpan, 1979; Inskeep and Hammerstedt, 1982) concentration of carnitine accompany epididymal transit. Immotile spermatozoa from the caput epididymis can be induced to express non-progressive movement if incubated in the presence of concentrations of carnitine which are found in the more distal portions of the epididymis (Hinton et al, 1981; Klinefelter and Hamilton, 1984). Furthermore both carnitine and acetylcarnitine stimulate sperm movement (Tanphaichitr, 1977), and the motility of mature spermatozoa is related to their carnitine content (Johansen and Bøhmer, 1979). As the motility of mature spermatozoa incubated in substrate free media is reduced, in parallel with a decline in pyruvate oxidation, by high concentrations of carnitine, but restored on the re-addition of substrate (Turner and Giles, 1981), it has been suggested that the main role of carnitine in spermatozoa is to provide substrate as acetylcarnitine. Exogenously added acetylcarnitine can be metabolised by spermatozoa (Storey and Keyhani, 1974; Hutson et al, 1977; Storey, 1980; Day-Francesconi and Casillas, 1982), and it has been shown that the acetylcarnitine/carnitine ratio is positively correlated with the motility of human spermatozoa (Golan et al, 1984). However, exogenous carnitine does not promote respiration in either immature (Casillas, 1973), or mature (Casillas, 1973; Johansen and Bøhmer, 1978) spermatozoa, nor does it stimulate ATP production in caput cells from the ram, whilst it does so in mature spermatozoa (Inskeep and Hammerstedt, 1982). It would thus seem that carnitine, although related to the acquisition of motility within the epididymis, is not a primary factor in this process.

Analysis of the literature reveals conflicting evidence concerning the effects of epididymal transit on the ATP content of spermatozoa. Concentrations of this nucleotide have been reported to increase (Chulavatnatol et al, 1977), or to remain constant (Ke and Tso, 1982) in rat spermatozoa; to rise in spermatozoa from the bull (Hoskins et al, 1975a), or to fall in the guinea pig and ram (Frenkel et al, 1973; Voglymayr, 1976). However, determination of the total cell content of ATP does not take into account possible changes in the sub-cellular distribution of this molecule.

The total ATPase activity expressed by spermatozoa has been found to decrease during their epididymal transit in rats (Chulavatnatol and Yindepit, 1976; Chulavatnatol et al, 1977; Majumder, 1981). In contrast, ATPase activity in both whole spermatozoa and isolated tails is known to increase during epididymal maturation in the ram (Voglmayr et al, 1969). However, the relationship between measurements of total ATPase activity and dynein ATPase activity is unknown.

ii. Protein carboxymethylation in the epidiymis. Protein carboxyl-methylase (PCM) transfers methyl groups from S-adenosyl-L-methionine to the carboxyl groups of glutamate and aspartate residues in methyl acceptor proteins (MAP) (Liss et al, 1969; Morin and Liss, 1973; Paik and Kim, 1980). Protein methylesterase (PME) hydrolyses the methyl ester bonds formed by PCM to yield methanol and the protein in its original, pre-methylation form (Stock and Koshland, 1978; Gagnon, 1979). This process of carboxy-methylation and demethylation is known to be involved in the control of flagellar movement in bacteria (Kort et al, 1975). The observation that carboxy-methylation of proteins was deficient in patients suffering from asthenozoospermia (Gagnon et al, 1980a; 1982), and that inhibitors of PCM activity, known to be localised in the sperm flagellum (Bouchard et al, 1980; Purvis et al, 1982), blocked sperm motility (Bouchard et al, 1981; Goh and Hoskins, 1985), suggest an involvement for this enzyme in the control of sperm movement (Gagnon et al, 1979, 1982). The increase in PCM activity and MAP capacity (Bouchard et al, 1980; Purvis et al, 1982), and the decrease in PME activity (Gagnon et al, 1984) which accompany epididymal maturation in the rat and bull further supports this theory. However, the reason why an increase in the methyl ester content of sperm proteins during epididymal maturation should increase motility is unkown, although effects on membrane fluidity (Hirata and Axelrod, 1978; Gagnon and Heisler, 1979) or calmodulin (Gagnon et al, 1980b) may be involved.

Protein carboxy-methylation also increases dramatically during the

capacitation of hamster spermatozoa (Castaneda et al, 1983), and inhibition of this process significantly depressed the expression of hyperactivation (Llanos and Meizel, 1983), although without any apparent effect on percentage motility (Meizel, 1981; Llanos and Meizel, 1983).

### iii. Protein phosphorylation in the epididymis.

a. Effects on mature sperm motility. Of all the compounds which have been linked with the spermatozoon's capacity for movement, possibly the most intensively studied has been cyclic adenosine 3',5' monophosphate (cAMP). The first evidence for an involvement of this agent in the control of sperm movement came from experiments which utilized methyl xanthines, particularly caffeine (1,3,7-trimethyl-2,6-dioxypurine), known to specifically inhibit the enzyme responible for cAMP's degradation, cyclic nucleotide phosphodiesterase (Butcher and Sutherland, 1962; Beavo et al, 1970). Using this experimental approach, it was shown that caffeine could stimulate the motility and respiration of caudal epididymal (Drevius, 1971, 1972; Garbers et al, 1971a; Hoskins, 1973) and ejaculated (Garbers et al, 1971b) spermatozoa from the bull, in association with the elevation of intracellular cAMP levels (Garbers et al, 1973a). Ejaculated human spermatozoa were also found to respond to caffeine in a similar manner (Bunge, 1973; Haesungcharern and Chulavatnatol, 1973; Schoenfeld et al, 1973, 1975; Homonnai et al, 1976; Chang and Boettcher, 1981). Other methylxanthine phosphodieterase inhibitors such as theophylline, aminophylline and pentoxifylline (Garbers et al, 1971a; Haesungcharern and Chulavatnatol, 1973; De Turner et al, 1978) and dibutryl cAMP (dbcAMP)(Garbers et al, 1971a), a membrane permeant analogue of cAMP, also increase the rates of respiration and motility in spermatozoa. In addition to the bull and the human, it has now been shown that the motility of spermatozoa from the pig (Garbers et al, 1973b) guinea pig (Frenkel et al, 1973b), hamster (Morton et al, 1974; Kann and Serres, 1980) and rat (Wyker and Howards, 1977; Paz et

al, 1978) is enhanced by treatment with phosphodiesterase inhibitors.

As human sperm metabolism was known to rely predominantly upon glycolysis (Peterson and Freund, 1968), and as this process was known to be greatly affected by cAMP (Hicks et al, 1972), it was thought that the effect of cAMP on motility may involve an increase in energy production by accelerating glycolysis and the tricarboxylic acid cycle (Haesungcharern and Chulavatnatol, 1973; Schoenfeld et al, 1975). However, since compounds like caffeine and theophylline affect other enzymes in addition to phosphodiesterase (Robison et al, 1971), it could not be conclusively demonstrated that these reagents were stimulating sperm motility by increasing cAMP content. Indeed, it has since been shown that caffeine stimulated motility in detergent treated bovine spermatozoa is not accompanied by an increase in cAMP dependant protein kinase activity (Tamblyn and First, 1977), and that the concentration of caffeine required to stimulate motility does not dramatically inhibit phosphodiesterase activity (Levin et al, 1981). Such results suggest that this particular compound was affecting sperm motility by means other than by the inhibition of phosphodiesterase activity.

More conclusive evidence was required to unequivocally link cAMP to sperm motility. To a certain extent, such evidence was provided by the demonstration that reversible inhibition of sperm motility, such as is produced upon slow cooling and rewarming, reversibly reduced the cAMP content of spermatozoa (Tash and Mann, 1973), whereas conditions which ireversibly inhibit motility, such as severe cold shock, produced irreversible reductions in cAMP levels (Tash and Mann, 1973; Chaudhry and Anand, 1975; Cascieri et al, 1976). Better evidence was provided by experiments in which cAMP was added to ATP reactivated, detergent lysed bovine sperm models. Such treatment produced measurable increases in both beat frequency and the proportion of motile cells (Lindemann, 1978), whilst demembranated trout and rat spermatozoa failed to initiate motility in the absence of cAMP (Morisawa and Okuno, 1982; Treetipasatit and Chulavatnatol, 1982). Further proof for the involvement of cAMP in the

capacity for movement were obtained from studies of the epididymal development of sperm motility as detailed below.

b. Involvement with epididymal acquisition of the capacity for movement. The acquisition of sperm motility in the epididymis was first linked to cAMP when the amount of this nucleotide present in spermatozoa was found to increase during epididymal transit (Hoskins et al, 1974). A direct causal relationship was thought to exist between the cAMP content of spermatozoa and their capacity for movement when it was found that treatment with phosphodiesterase inhibitors induced non-progressive flagellar movement in previously immotile spermatozoa from the bovine caput epididymis (Hoskins et al, 1975b). This motion could be converted into progressive motility by the addition of forward motility protein (see above). However, testicular spermatozoa from the ram (Hammerstedt and Hay, 1980) and bull (Cascieri et al, 1976) did not show an increase in motility in response to elevations of their cAMP content, implying that in these species the ability to respond to cAMP also develops during epididymal maturation. This may involve protein kinase activity, which is known to increase in bovine spermatozoa as they progress through the epididymis (Hoskins et al, 1974; Pariset et al, 1985). A similar increase in cAMP content was reported for ram spermatozoa (Amann et al, 1982), although a subsequent study has failed to support this finding (Pariset et al, 1985). In the rat, however, a decrease in cAMP content has been reported to accompany epididymal maturation (Del Rio and Raisman, 1978).

## E. Mechanisms for the suppression of sperm motility

Morton et al, (1974) reported that the initiation of hamster caudal sperm motility in response to dilution was dependant upon the presence of calcium within the media. Calcium's effects on motility were thought to be

due to a stimulation of adenylate cyclase activity, as the cAMP content of these spermatozoa rose rapidly in response to dilution. This group postulated that these spermatozoa were maintained in an immotile condition during storage in the cauda epididymis via the action of a phosphodiesterase present in caudal epididymal fluid (CEF), which kept their endogenous cAMP content low. A similar increase in cAMP content was reported to accompany dilution of bovine spermatozoa and to precede the expression of movement by these cells (Cascieri et al, 1976), although motility initiation in this species did not require the presence of calcium. In the light of this and a subsequent study which showed that the dilution of rabbit caudal epididymal spermatozoa in aerated saline was sufficient to initiate motility (Storey, 1975), Morton et al (1978) forwarded the hypothesis that the degree of motility expressed within the epididymis of different species was related to the free calcium content of the epididymal plasma, and that an inverse relationship existed between the free calcium content of CEF and the extent to which sperm motility could be induced by calcium. However, it has since been shown that the induction of motility in spermatozoa from rats (Turner and Howards, 1978; Chulavatnatol, 1982; Usselman and Cone, 1983; Turner and Reich, 1985) and hamsters (Turner and Reich, 1985), both of which should have the strongest calcium requirement for motility initiation according to the theory of Morton et al (1978), was not dependant upon the presence of this cation.

Following the observation that dilution of bovine CES in CEF was sufficient to initiate progressive motility (Cascieri et al, 1976), this group proposed that CEF from this species did not contain a motility inhibiting factor, but that the partial quiescence enforced upon these cells whilst resident in the male tract was due to cell/cell contact. However, later studies did not confirm these findings (Carr and Acott, 1984; Acott and Carr, 1984).

Carnitine and glycerophosphorylcholine, both of which are present in CEF (Brooks et al, 1974; Hinton and Setchell, 1980a,b; Casillas, 1973; Frenkel et al, 1974), have also been implicated as the motility inhibitor in epididymal fluid, although in these cases also, further investigations failed to

substantiate these claims (Turner et al, 1978; Turner and Giles, 1981; Hinton et al, 1981).

Two contrasting methods for maintaining sperm quiesence have been identified and verified for spermatozoa of different species. Following the demonstration that the dilution of rat CES in rat CEF did not result in the induction of motility (Turner and Howards, 1978), it was further shown that the sperm-immobilising capacity of rat CEF was abolished following treatment with proteases (Turner and Giles, 1982), indicating that an epididymal protein was required for inhibition of motility. These findings were confirmed and extended by Usselman and Cone (1983), who isolated a high molecular weight, mucus-like glycoprotein from rat CEF, which they named "Immobilin". They hypothesised that this molecule inhibited epididymal sperm movement mechanically, via the creation of a highly viscoelastic environment. A similar situation has been found to exist in the hamster (Turner and Reich, 1985). However, in the bull, although vigorous motility is initiated in a wide variety of ionic and non-ionic, osmotically-balanced buffers (Carr and Acott, 1984), CEF from this species has a low viscoelasticity. Following the observation that the pH of bovine CEF is 5.8 (Acott and Carr, 1984), it was proposed that the inhibition of CES motility in this species was due to a pH-dependant factor present in CEF (Acott and Carr, 1984). A similar mechanism appears to operate in the dog (Carr et al, 1985). However, CES from the guinea pig and the human, which have neither viscoelastic nor acidic CEF, are immotile whilst in the cauda epididymis and show no specific ionic requirement for the initiation of motility (Turner and Reich, 1985; Carr et al, 1985). The mechanism by which spermatozoa from these species are maintained in a quiesence state remains to be elucidated.

The quiescence of spematozoa in the isthmus of the oviduct may be enforced by a putative molecular motility inhibitor (Suarez 1987), or by local chemical or temperature differences in this region of the female tract (Hunter and Nichol, 1986). The possibility exists that the storage function of the distal Mullerian duct (oviduct isthmus, and the distal Wolffian duct (cauda

epididymis) may have a common physiological basis (Hunter and Nichol, 1983, 1986; Suarez, 1987).

## F. Mechanisms for the generation of hyperactivated motility

Little is known of the mechanisms by which spermatozoa begin hyperactivated motility. Lectin studies have revealed changes in the exposed carbohydrates on the flagellar membrane during capacitation (Kinsey and Koehler, 1978; Talbot and Chacon, 1981; Ahuja, 1984, 1985), whilst Koehler and Gaddum-Rosse (1975) and Friend et al (1977) have reported changes in the pattern of distribution of intramembranous particles in the plasma membrane overlying the midpiece prior to the expression of hyperactivated motility by guinea pig spermatozoa. The significance of these changes are unknown, although they may indicate alterations in ion fluxes across the membrane, as a specialised region of intramembranous particles (the"zipper") is thought to be a site of ion exchange (Friend and Heuser, 1981). Furthermore, both sperm motility and particle dispersion are inhibited by cyanide (Friend, 1977), although in view of the pernicious nature of this compound, these effects may only be coincidental. The stimulation of hyperactivation by membrane active agents (Yanagimachi, 1975) and lysophospholipids (Fleming and Yanagimachi, 1981), and the inhibition of this movement pattern by phospholipase blockers (Llanos and Meizel, 1983) further suggests the involvement of membrane changes in the induction of hyperactivated motility.

The expression of hyperactivated motility by intact spermatozoa is known to depend upon the presence of calcium (Yanagimachi and Usui, 1974, Fraser, 1977, Shams-Borhan and Harrison, 1981; Yanagimachi, 1982), and in the mouse at least, glucose (Fraser and Quinn, 1980; Cooper, 1984). Cyclic AMP has also been implicated in the induction of hyperactivation, as both caffeine and dibutryl cAMP advance its onset in mouse and hamster spermatozoa (Fraser, 1977, 1981; Mrsny and Meizel,

1980)

The observation that detergent permeabilised hamster, guinea pig and human spermatozoa express hyperactivated motility immediately upon reactivation with ATP alone (Mohri and Yanagimachi, 1980), suggests that the appearance of this flagellar waveform is actively suppressed in uncapacitated spermatozoa. Furthermore, spermatozoa from the proximal cauda epididymis of the hamster express hyperactivation immediately upon dilution (S Suarez, personal communication), suggesting that some factor in the plasma from the distal cauda epididymis prevents spermatozoa from expressing hyperactivation.

## G. Aim of this study

In view of the evidence sited above, it appears that cAMP may play a pivotal role in the acquisition of the capacity for movement which occurs during epididymal maturation, in the initiation of motility upon ejaculation, and in the post-ejaculatory modifications of sperm-movement which occur during capacitation in the female genital tract. This study sets out to investigate the involvement of cAMP in these post-testicular modifications of sperm movement using the hamster as an animal model.

# Chapter 2. The influence of epididymal maturation on the cAMP levels in hamster and rat spermatozoa.

## A. Introduction.

Mammalian spermatozoa isolated from the rete testis show only uncoordinated twitching movements upon dilution. The ability to initiate progressive motility is gradually attained during passage through the caput and corpus epididymis, and spermatozoa which have completed epididymal maturation are then stored in a quiescent state in the cauda epididymis prior to release at ejaculation, at which time vigorous motility is expressed.

The exact biochemical mechanisms involved in the development and expression of sperm motility remain unknown. However, a pivotal role has been implicated for cyclic 3',5'-adenosine monophosphate (cAMP). The motility of mature spermatozoa is enhanced by treatment with phosphodiesterase inhibitors, membrane permeant analogues of cAMP and stimulators of adenylate cyclase (Garbers and Kopf, 1980; Tash and Means, 1983; Vijayaraghavan and Hoskins, 1986; Aitken et al, 1986), whilst the flagellar beat of both mature and immature detergent-permeabilized, ATP-reactivated spermatozoa is stimulated by the addition of cAMP to the reactivation medium (Lindemann, 1978; Mohri and Yanagimachi, 1980; Treetipasatit and Chulavatnatol, 1982; Tash and Means, 1982; Yeung, 1984). In the bull (Hoskins et al, 1974), and the ram (Amann et al, 1982), the development of the potential for movement which occurs during epididymal passage is correlated with a rise in sperm cAMP levels. Furthermore, coordinated flagellar movement can be induced in intact caput spermatozoa by treatment with phosphodiesterase inhibitors (Hoskins et al, 1975b; Kann and Serres, 1980). In addition, several groups have proposed that the initiation of motility which occurs upon dilution of caudal spermatozoa is mediated by an increase in cAMP levels (Morton et al, 1974; Cascieri et al, 1976; Okumura et al, 1985). Coupled with the observation that the expression of flagellar movement in a number of different motile systems is known to require the cAMP-dependant phosphorylation of a 56 Kd axonemal protein named axokinin (Tash et al, 1984), the hypothesis that an increase in cAMP levels during epididymal maturation may be directly

involved in the acquisition of sperm movement is attractive. However, preliminary observations in the rat have shown that epididymal transit in this species is accompanied by a decrease in sperm cAMP content (Del Rio and Raisman, 1978). In the light of this discrepancy, this study has analysed the effects of epididymal maturation on the cAMP content of rat and hamster spermatozoa.

## B. Materials and Methods.

i. Media. The basic medium used throughout this study was a modified Tyrodes solution (MT-1). This contained 125mM NaCl, 2.7mM KCl, 1.8mM $CaCl_2$, 0.5mM $MgCl_2$, 0.36mM $NaH_2PO_4$, 11.9mM $NaHCO_3$, 4.5mM glucose, 0.09mM pyruvate, 8.9mM sodium lactate, 0.5mM taurine, and 3mg/ml bovine serum albumin (BSA-RIA grade); 1.0ml penicillin streptomycin (Difco laboratories, Detroit, Michigan, U.S.A.) was added to each 100ml of media. Additional NaCl was added to MT-1 containing no added calcium (NAC) in order to maintain constant osmolarity (approx. 290 mOsm). Under such circumstances, the free calcium concentration of this medium was determined to be 10 μM (measured using a calibrated Orion calcium-sensitive electrode).

Rat spermatozoa were incubated in the medium used by Toyoda and Chang (1974). This contained 94.6mM NaCl, 4.78mM KCl, 1.71mM CaCl, 1.19mM $KH_2PO_4$, 1.19mM $MgSO_4$ and 25.07mM $NaHCO_3$, to which 21.58mM sodium lactate, 0.5mM sodium pyruvate, 5.56 mM glucose, 4mg/ml BSA were added; Each 100ml of media was treated with 1.0ml penicillin-streptomycin.

For media preparation, inorganic salts were dissolved in double distilled water and stored in a refrigerator. Phenol red (100μl, 0.5% solution - Flow laboratories, Irvine, Scotland) was added to these stock solutions as a pH indicator. The other components were dissolved in this stock immediately prior to use, with fresh media being made up each day. Medium MT-1 was equilibrated by bubbling 5% $CO_2$ in air through it prior to the addition of BSA. All incubations were performed at 37°C in an atmosphere of 5% $CO_2$:95% air. Unless otherwise indicated, biochemicals were obtained from either Sigma (Dorset, UK) or BDH (Glasgow, UK).

ii. Isolation of Spermatozoa. Adult male Golden Syrian hamsters (< 100g) were asphyxiated using $CO_2$ and killed by cervical dislocation. Their

epididymides were quickly excised and separated into caput and caudal regions. These organs were then transferred to moist filter paper on a warm stage maintained at 37°C, where adherent adipose tissue was removed. Organs were blotted dry prior to isolation of spermatozoa. Caput epididymides were punctured in the proximal portion (site 2 - Hinton et al, 1979a) by several passes of a #26 guage needle and spermatozoa isolated by gentle compression using broad tipped forceps. Caudal epididymal spermatozoa were extruded under positive pressure from the cut ends of 2 or 3 exposed tubules from the distal portion (site 7 - Hinton et al, 1979) of this organ.

For experiments requiring undiluted cells ($T_o$ determinations), spermatozoa were drawn into 20µl microcapillary tubes (Drummond Scientific, Pennsylvania, U.S.A.). These were plugged at one end with critaseal (Hawksley & Sons LTD, Sussex, England) and centrifuged at 200g for 5 mins. After centrifugation, the height of the sample within the tube was carefully measured and compared to known values for packed sperm density in these tubes. These values were pre-determined for the different cell types by expressing measured "lengths" of spermatozoa into a known volume of media and estimating the sperm density using an improved Neubauer haemocytometer chamber. With the knowledge that a 1cm length of these 20µl microcapillary tubes contains a volume of 2.94µl, the following values were obtained:

Hamster caput fluid sperm density = $3.12 \times 10^6$/cm = $1.06 \times 10^6$/µl.

Hamster cauda fluid sperm density = $3.12 \times 10^6$/cm = $1.06 \times 10^6$/µl.

Rat caput fluid sperm density = $1.59 \times 10^6$/cm = $5.41 \times 10^5$/µl.

Rat cauda fluid sperm density = $3.70 \times 10^6$/cm = $1.26 \times 10^6$/µl.

These values are in close agreement with those determined in previous studies for the hamster (Jesse and Howards, 1976) and rat (Turner et al, 1977, 1984; Turner and Cesarini 1983; Turner, 1984).

For experiments performed on diluted spermatozoa, epididymides

were prepared as above, then placed under pre-warmed and $CO_2$ equilibrated liquid paraffin in plastic tissue culture dishes (60x15mm, Becton Dickinson, NJ, USA) on a binocular microscope stage maintained at a temperature of 37°C. The spermatozoa were extruded as detailed above, then gently eased across the dish into a 0.5ml (caput) or 1.0ml (cauda) droplet of medium, using a preformed hooked rod fashioned from an extruded glass pasteur pipette. This dilution event was defined as $T_0$. Spermatozoa were then dispersed by gentle agitation of the petri dish. After even dispersion had occured (approx. 1 min), a 10µl sample was removed and counted using a haemocytometer chamber. The concentration of spermatozoa was then adjusted to the required density by appropriate dilution. The accuracy of this adjustment was checked by determining the cell concentration after dilution. Preparations of caput spermatozoa were determined to contain less than 1% contamination with other cell types, whilst incubations of caudal spermatozoa were adjudged to be pure.

**iii. Cyclic AMP Extraction.**

a. Undiluted spermatozoa. For undiluted samples, the microcapillary tubes containing the spermatozoa were broken at one end after mensuration and the contents extruded into 500µl of ice cold ethanol (Aitken et al, 1986). Spermatozoa treated in this manner tended to clump into globules rather than to disperse freely throughout the liquid. In order to check whether efficient extraction of cAMP was being achieved, especially from cells in the centre of a clump, several samples were split and either extracted as normal, or homogenised in a 0.1ml Uni-Form hand held homogeniser (Jencons Ltd, Leighton Buzzard, UK). No significant difference was found between the cAMP content measured by these different methods, and it was thus felt unneccesary to homogenise undiluted spermatozoa during cAMP extraction. Extraction procedures following immersion in ethanol were as detailed below for diluted spermatozoa.

b. Diluted spermatozoa. For incubations performed at $20x10^6$/ml, two 50µl aliquots were removed at various time intervals and placed immediately into 500µl of ice cold ethanol. For experiments utilising spermatozoa at a density of $1x10^6$/ml, three 1.0ml samples were removed from the incubation and centrifuged at 250g for 5 min. After removal of the supernatant, two of the pellets were resuspended in ethanol, whilst the third was resuspended in 950µl of media. The density of spermatozoa in this tube was then determined to account for losses during centrifugation.

After 20 mins in ethanol, cellular material was separated by centrifugation (2000g, 10 min) and the supernatant decanted into flat bottomed glass tubes and dried down under a stream of nitrogen at 40°C. The residue was reconstituted in 500µl of the assay buffer {Phosphate buffered saline (PBS), 0.01mM, pH7.4} and stored at -17°c until assayed.

iv. Cyclic AMP Determination. The cAMP content of the extracted samples was assessed according to the method of Steiner et al (1972) as modified by Harper & Brooker (1975). 100µl duplicates from each sample were treated with 5µl of freshly prepared acetylating reagents (2.7 parts triethylamine: 1 part acetic anhydride), mixed and incubated at room temperature for 1h. Subsequently, 100µl antiserum (1:70 000 dilution made up in assay buffer containing 0.3% bovine gamma globulin) raised against a conjugate of human serum albumin and succinyl cyclic AMP (Steiner et al., 1972), and 100µl (30000 cpm) $^{125}$I-2'0-succinyl cAMP-tyrosine methyl ester tracer (Sigma Chemical Co, St Louis, MO, U.S.A.) were added to each tube and incubated for 16 h at 4°C; cAMP standards (0-2500 fmol) were treated in precisely the same way. The bound complexes were precipitated by the addition of 1.6ml of ice cold 17% (w/v) polyethylene glycol, separated by centrifugation (2000g for 30 min at 4°C) and the radioactivity associated with the pellets counted using a Nuclear Enterprises 1600 multihead gamma counter. The amount of cAMP present in each sample was calculated by computer, relative to a log-logit standard curve constructed for each assay,

and expressed as pmol per $10^9$ spermatozoa. The cross reactivities of the antibody used in these assays are: cAMP, 100%; cGMP, 0.0035%; AMP, 0.0005%; ADP, 0.00045%; ATP, 0.00069%; GTP, 0.0005%; isobutyl methyl xanthine, 0.00001%. Media blanks did not cross react in this assay. The intra - and inter-assay coefficients of variation were 6.3% and 14.5% respectively.

v. Statistics. All statistical comparisons were made using the non-parametric Mann-Whitney U test. Unless otherwise indicated, values shown represent the mean of six separate determinations ± the standard error of the mean.

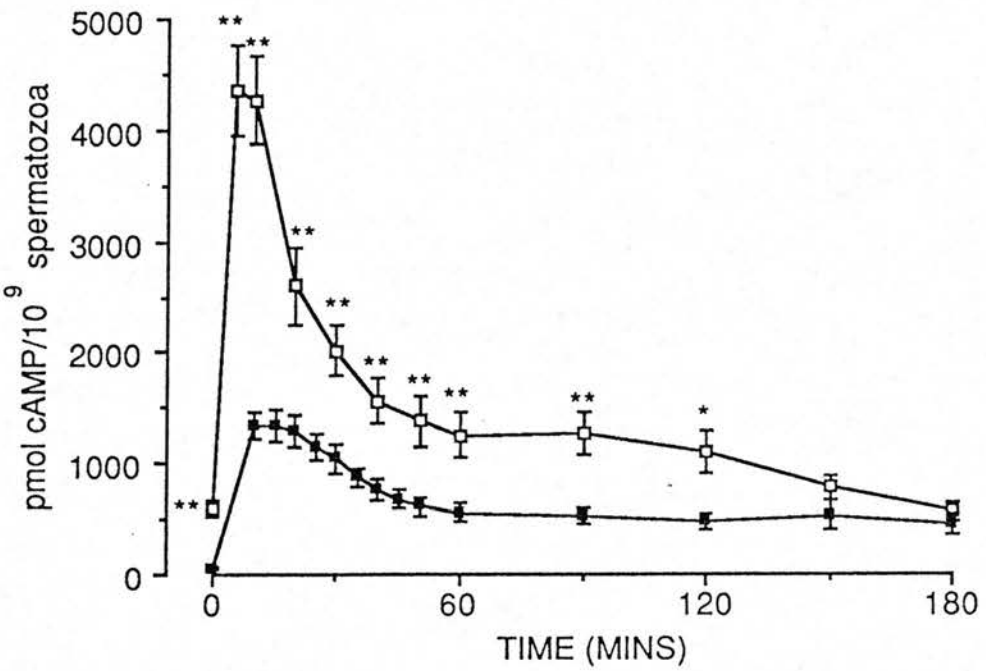



Figure 4. Cyclic AMP content (picomoles cAMP per $10^9$ spermatozoa) expressed by spermatozoa from the hamster caput (open squares) or cauda (closed squares) epididymis incubated over a 3h period in medium MT-1 at a density of $20x10^6$/ml. Significance levels are: *, $P<0.05$; **, $P<0.01$. Values shown are mean ± standard error, n=6.

## C. Results.

### i. Cyclic AMP

a. Basal levels in undiluted spermatozoa. Whilst still in the fluid environment of the epididymis, hamster caput spermatozoa were found to contain significantly ($P<0.01$) greater amounts of cAMP than caudal spermatozoa {597.4 ± 81.0 (n=9) vs 56.0 ± 6.2 (n=14) pmol cAMP/$10^9$ spermatozoa (mean ± s.e.m.)}. Similarly, spermatozoa isolated, without dilution, from the caput epididymis of the rat were found to contain significantly ($P<0.01$) greater amounts of cAMP than caudal cells {176.5 ± 26.0 (n=11) vs 44.1 ± 11.3 (n=11) pmol cAMP/$10^9$ spermatozoa (mean ± sem)}

### b. Response to dilution at $20x10^6$/ml.

### 1. In complete medium (MT-1).

A. Hamster. Spermatozoa from both the caput and cauda epididymis exhibit an initial rapid increase in cyclic AMP content in response to dilution in MT-1 at a density adjusted to $20x10^6$/ml. However, this increase is not identical for both cell types, as the cAMP content of caput spermatozoa is elevated to significantly ($P<0.01$) higher levels than that of caudal spermatozoa. The cAMP content of spermatozoa from both these regions of the epididymis subsequently decayed over time, such that after 150min of incubation, the cAMP content of caput and caudal cells was not significantly different (Fig 4.)

B. Rat. Rat spermatozoa from both the caput and cauda epididymis exhibited a rapid increase in cAMP content upon dilution into T&C at a density adjusted to $20x10^6$/ml, in a manner similar to that seen in

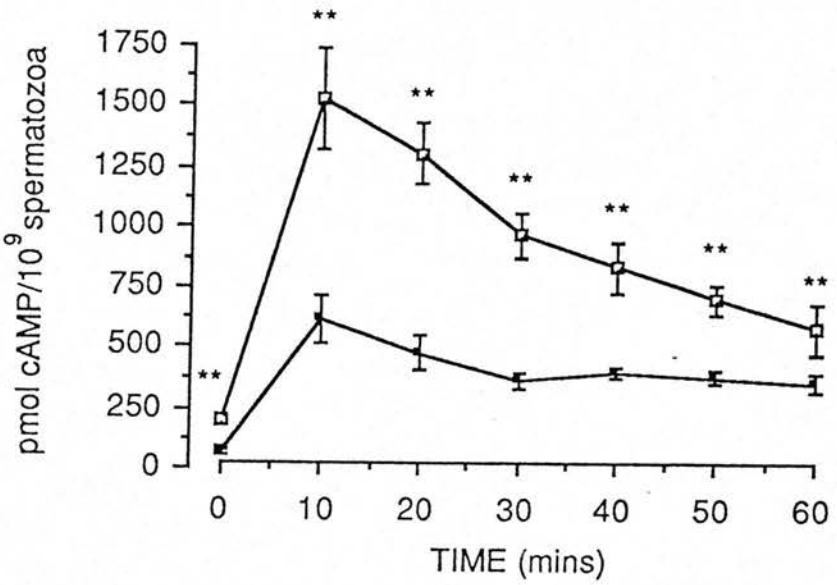



Figure 5. Cyclic AMP content (picomoles cAMP per $10^9$ spermatozoa) expressed by spermatozoa from the rat caput (open squares) or cauda (closed squares) epididymis incubated over a 60min period in medium T&C at a density of $20x10^6$/ml. Significance levels are: **, $P<0.01$. Values shown are mean ± standard error, n=6.

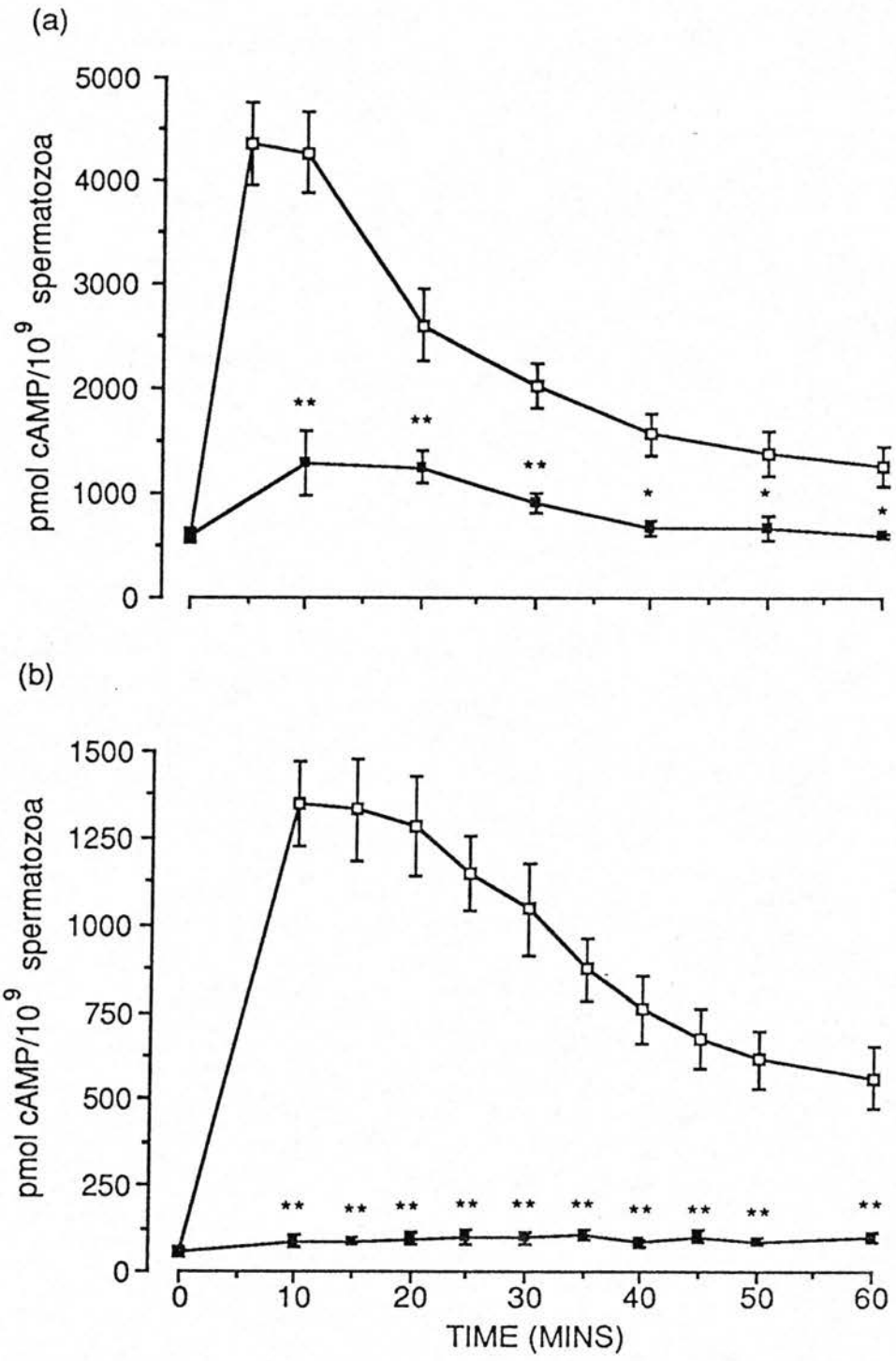



Figure 6. Cyclic AMP content (picomoles cAMP per $10^9$ spermatozoa) expressed by spermatozoa from the hamster caput (a), or cauda (b) epididymis incubated over a 60min period at a density of $20x10^6$/ml in medium MT-1 (open squares), or medium NAC (closed squares). Significance levels are: *, $P<0.05$; **, $P<0.01$. Values shown are mean ± standard error, n=6.

spermatozoa from the hamster (see above). Caput spermatozoa expressed significantly ($P<0.01$) higher levels of cAMP than spermatozoa from the cauda epididymis (Fig 5). The cAMP content of both caput and caudal cells subsequently decreased after this initial peak, but after 60mins of incubation, caput levels were still significantly ($P<0.01$) greater than in caudal spermatozoa. At this time point, the cAMP content of both cell types were also still significantly ($P<0.01$) greater than in undiluted spermatozoa.

For further characterization of the increases in cAMP content detailed above, it was decide to utilize the hamster as an animal model. The results detailed hereafter thus refer only to spermatozoa from this species.

2. In medium NAC. Release of caput spermatozoa from the hamster into medium MT-1 devoid of exogenously added calcium (NAC), at a cell concentration adjusted to $20x10^6$/ml, caused a significant inhibition ($P<0.01$) of the rise in cAMP levels observed upon dilution into complete medium MT-1 (Fig 6a). However, a statistically significant ($P<0.05$) increase in cAMP content over the levels in undiluted spermatozoa was still apparent. In contrast, the rise that accompanied dilution of caudal spermatozoa into MT-1 was completely abolished by release into NAC (Fig 6b).

The reduction of cAMP levels which resulted from the removal of exogenously added calcium from the media could result from either an inhibition of the synthesis, or enhancement of the degradation of cAMP. To determine which of these possibilities was occuring, both caput and caudal spermatozoa were incubated in either medium MT-1, or medium NAC in the presence or absence of the phosphodiesterase inhibitor 3-isobutyl-1-methyl-xanthine (IBMX) at a concentration of 0.2mM. This reagent caused a significant ($P<0.01$) stimulation of the cAMP levels in both caput and caudal spermatozoa, regardless of the calcium content of the media. However, the levels expressed following this treatment with IBMX were significantly ($P<0.01$) lower in medium NAC than in medium MT-1(Fig 7).

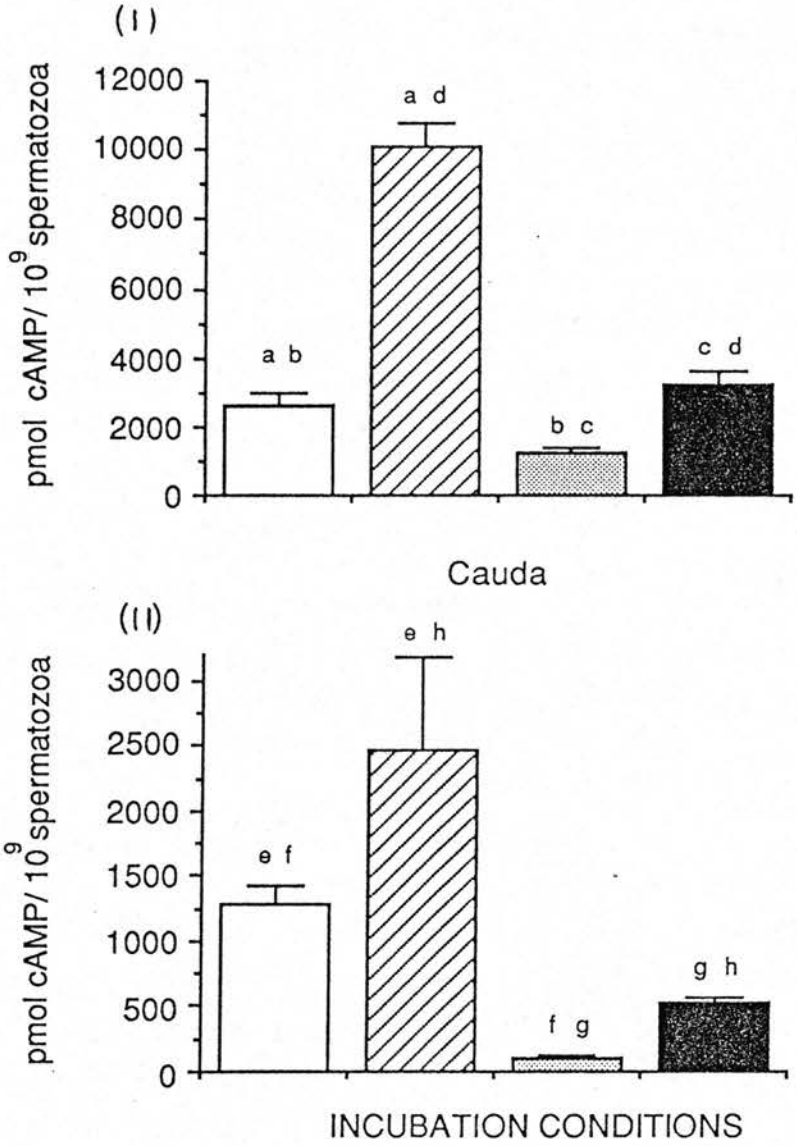



Figure 7. Cyclic AMP content (picomoles cAMP per $10^9$ spermatozoa) expressed by spermatozoa from the hamster caput (I), or cauda (II) epididymis incubated for 20mins at a density of $20x10^6$/ml in: medium MT-1 (open bars), medium MT-1 plus 0.2mM IBMX (lined bars), medium NAC (dotted bars), or medium NAC plus 0.2mM IBMX (solid bars). Treatment groups bearing the same letter are significantly ($P<0.01$) different. Values shown are mean ± standard error, n=6.

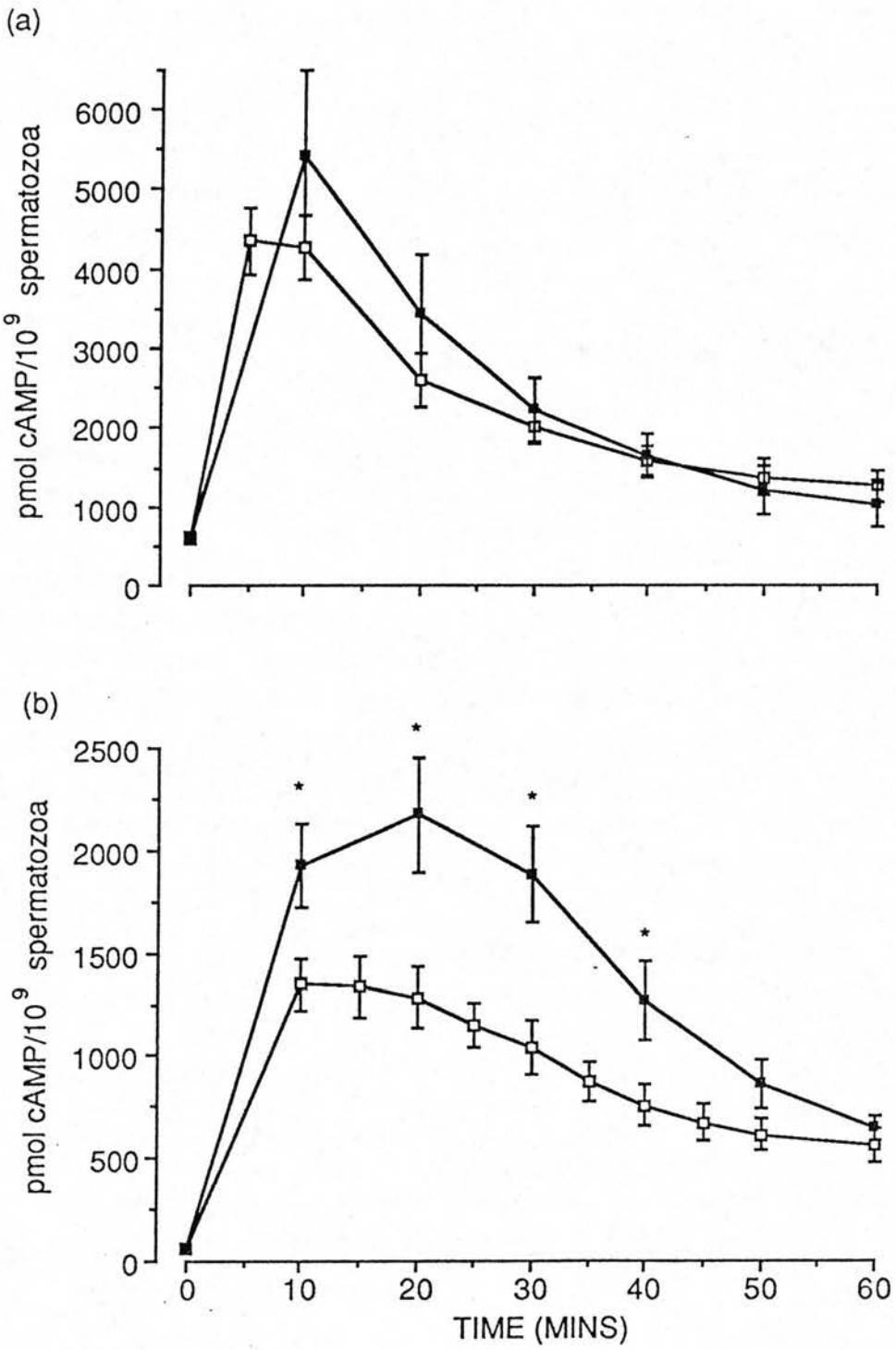



Figure 8. Cyclic AMP content (picomoles cAMP per $10^9$ spermatozoa) expressed by spermatozoa from the hamster caput (a), or cauda (b) epididymis incubated over a 60min period at a density of $20x10^6$/ml in medium MT-1 (open squares), or medium MT-1 plus 0.5μM calmidazolium (closed squares). Significance levels are: *, $P<0.05$. Values shown are mean ± standard error, n=6.

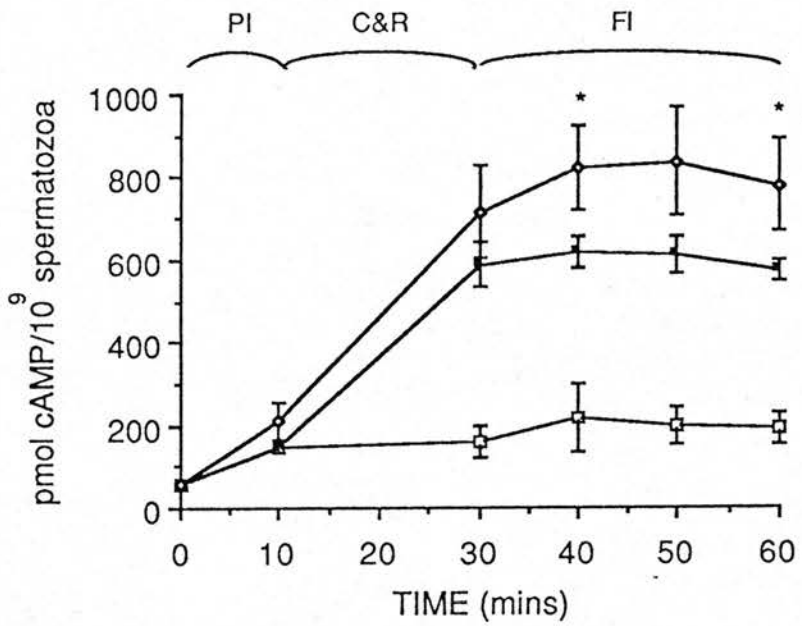



Figure 9. Cyclic AMP content (picomoles cAMP per $10^9$ spermatozoa) expressed by spermatozoa from the hamster cauda epididymis, at a density of $20x10^6$/ml, pre-incubated (PI) for 10mins in either medium NAC (closed squares), or medium NAC plus 0.5µM calmidzolium (open diamonds), then centrifuged and resuspended (C&R) in; medium NAC (open squares), medium MT-1 (closed squares), or medium MT-1 plus 0.5µM calmidzolium (open diamonds) for a final incubation (FI) period of 30mins. Significance levels are: *, $P<0.05$. Values shown are mean ± standard error, n=6.

3. In the presence of calmodulin inhibitor. In view of previous observations which reported that increases in the cAMP content of guinea pig spermatozoa were dependant upon the action of calmodulin (Hyne and Garbers, 1979a), it was decided to determine whether the calcium dependant rise in cAMP levels observed upon dilution in spermatozoa from the hamster caput and cauda epididymis was mediated by this calcium binding protein. This was achieved using the calmodulin antagonist, calmidazolium (CZ, compound R 24571), at a concentration of 0.5μM, known to be optimal for the inhibition of other calmodulin dependant enzymes (Geitzen et al, 1982).

In contrast to our expectations, when calcium and CZ were presented to spermatozoa simultaneously, by releasing the cells directly into MT-1, both caput and caudal spermatozoa exhibited an increase in cAMP content, although this rise was significant ($P<0.05$) only for caudal spermatozoa (Fig 8). Pre-incubation of caudal spermatozoa in NAC plus CZ, followed by resuspension of these cells in MT-1 plus CZ similarly induced a significant ($P<0.05$) increase in the cAMP content of caudal cells compared to the levels of this nucleotide expressed by spermatozoa preincubated in NAC and resuspended in MT-1 (Fig 9).

4. In the presence of phospholipase A2 inhibitor. As changes in membrane phospholipid content are known to affect adenylate cyclase activity (Houslay et al, 1976; Bakardjiera et al, 1979; Salesse and Garnier, 1984), and hamster spermatozoa have been shown to exhibit phospholipase A2 activity (Llanos et al, 1982). the effects of the phospholipase A2 inhibitor -p-dibromoacetophenone on the cAMP content of caput and caudal spermatozoa were determined. At 20μM, a dose known to inhibit this enzymes activity in spermatozoa of other species (J Crosby and RW Kelly, personal communication), this reagent had no significant effect on the cAMP levels expressed by either cell type (Fig 10).

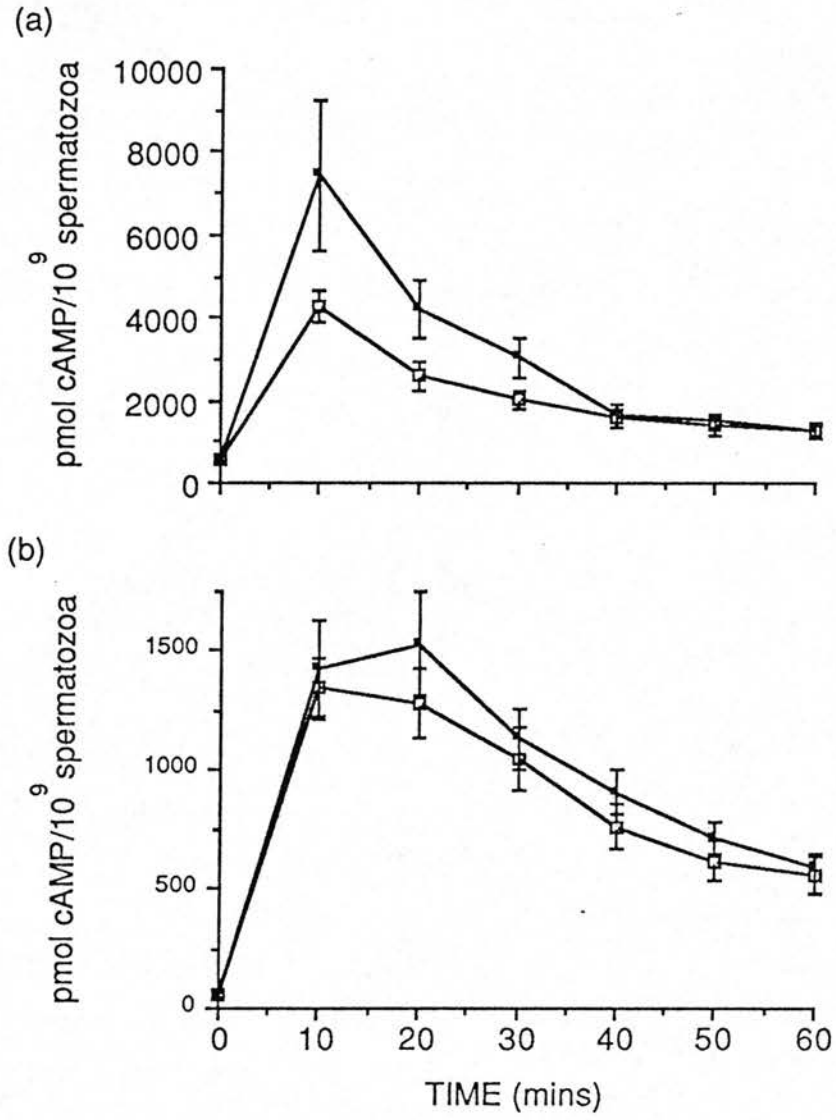



Figure 10. Cyclic AMP content (picomoles cAMP per $10^9$ spermatozoa) expressed by spermatozoa from the hamster caput (a), or cauda (b) epididymis incubated over a 60min period at a density of $20x10^6$/ml in medium MT-1 (open squares), or medium MT-1 plus 20μM -p-dibromoacetophenone (closed squares). Values shown are mean ± standard error, n=6.

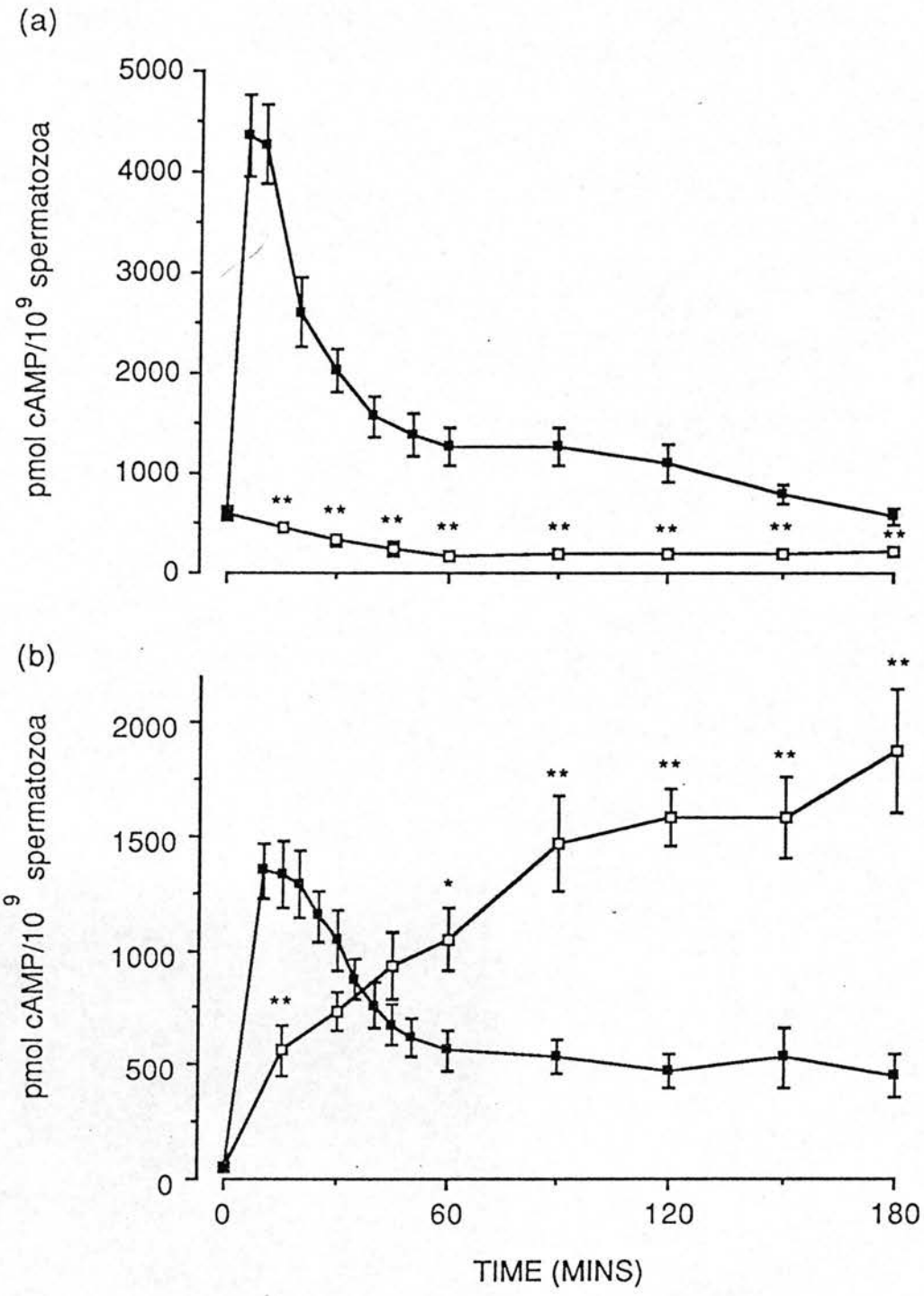



Figure 11. Cyclic AMP content (picomoles cAMP per $10^9$ spermatozoa) expressed by spermatozoa from the hamster caput (a), or cauda (b) epididymis incubated over a 3h period in medium MT-1 at a density of $20x10^6$/ml (closed squares), or $1x10^6$/ml (open squares). Significance levels are: *, $P<0.05$; **, $P<0.01$. Values shown are mean ± standard error, n=6.

c. Response to dilution at 1x10^6/ml. Caput and caudal spermatozoa released into MT-1 and incubated at a density adjusted to $1x10^6$/ml showed completely different cAMP responses from those observed in incubations performed at a cell concentration of $20x10^6$/ml. The cAMP content of caput spermatozoa fell from the basal undiluted level and remained low throughout the incubation, in marked contrast to the dramatic increase in cAMP levels seen at $20x10^6$/ml (Fig 11a).

The initial rise in cAMP content expressed by caudal spermatozoa in response to dilution at $20x10^6$/ml was also significantly ($P<0.01$) reduced in incubations performed at the lower density of $1x10^6$/ml [1334 ± 147 vs 565 ± 110 pmol cAMP/ $10^9$ spermatozoa, 15min after dilution]. However, spermatozoa from this portion of the epididymis show a more gradual, progressive, time-dependant increase in cAMP content, with the intracellular concentration of this nucleotide reaching significantly ($P<0.01$) higher levels than in undiluted spermatozoa after 15 min of incubation. At this lower density, the cAMP content of caudal spermatozoa eventually reached levels significantly higher than those shown at $20x10^6$/ml (Fig 11b).

d. Epididymal plasma effects on cAMP content of caput spermatozoa. The dramatic fall in the cyclic AMP content of caput epididymal spermatozoa on dilution from $20x10^6$/ml to $1x10^6$/ml may have been caused by the loss of key factors in the epididymal fluid, which become diluted out when spermatozoa are prepared at the lower density. To investigate this possibility, the influence of conditioned media recovered from suspensions of caput spermatozoa at a density of $20x10^6$/ml on the cAMP content of the same cell type at a concentration of $1x10^6$/ml was determined.

The conditioned medium was prepared by releasing caput spermatozoa into medium MT-1 at a cell density adjusted to $20x10^6$/ml. These spermatozoa were then pelleted by centrifugation at 250g for 5 min. The supernatant was removed, frozen and stored at -20°C until required for

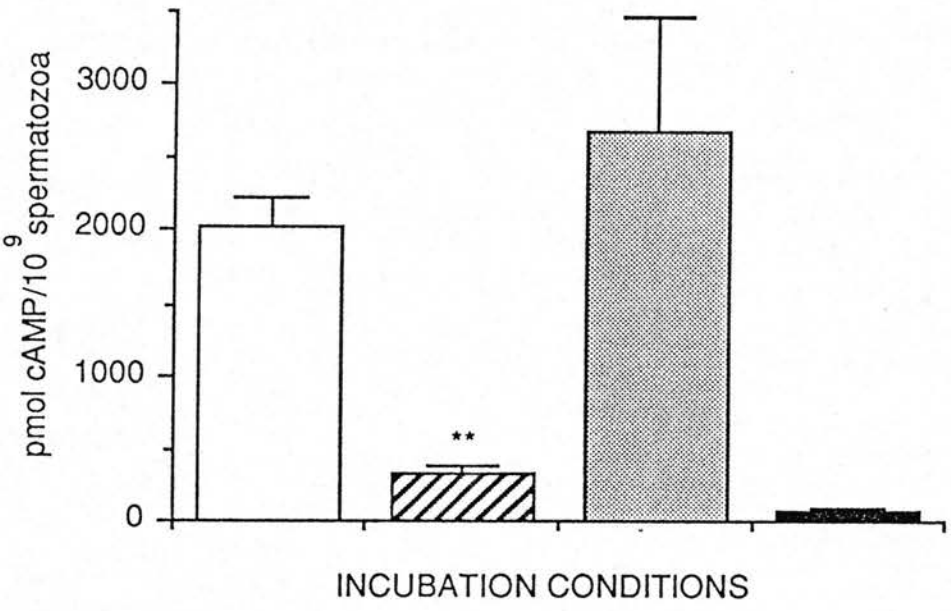



Figure 12. Cyclic AMP content (picomoles cAMP per $10^9$ spermatozoa) expressed by spermatozoa from the hamster caput epididymis incubated for 30min under the following conditions:

A. Open bar - At a density of $20x10^6$/ml in medium MT-1,

B. Lined bar - At a density of $1x10^6$/ml in medium MT-1,

C. Dotted bar - At a density of $1x10^6$/ml in the 250g supernatant from incubation A. The closed bar represents the cAMP content of the supernatant from incubation A alone.Significance levels are: **, $P<0.01$. Values shown are mean ± standard error, n=6.

use, at which time it was thawed and rewarmed to 37°C. Aliquots of fresh MT -1 were treated in the same manner to act as control media. Caput spermatozoa released into this epididymal plasma conditioned media at a density adjusted to $1x10^6$/ml, expressed cAMP levels of 2671 ± 776 pmol cAMP/$10^9$ cells after 30 mins of incubation, significantly ($P<0.01$) greater than the cAMP content of these cells incubated at the same density in control MT-1 (331 ± 58 pmol cAMP/$10^9$ spermatozoa). However, the cAMP content of spermatozoa in conditioned media did not differ significantly from the levels expressed by caput spermatozoa incubated in medium MT-1 at a density of $20x10^6$/ml (2018 ± 220 pmol cAMP/$10^9$ spermatozoa) (Fig 12). The conditioned medium itself contained only a small amount of cAMP. This was attributed to the presence of a small number of spermatozoa which had not been removed by centrifugation.

e. Phosphodiesterase inhibitor effects on cAMP content of caput spermatozoa. Caput spermatozoa incubated at a density of $1x10^6$/ml do not exhibit the rise in cAMP levels which is expressed by these cells upon incubation at a concentration adjusted to $20x10^6$/ml (Fig 11a). The results presented above suggest that this may result from the dilution of some factor present in epididymal plasma (Fig 12). To investigate the possibilty that such a factor may be a phosphodiesterase inhibitor (Benau et al, 1986), caput spermatozoa were incubated in MT-1 at a density of $1x10^6$/ml in the presence of the synthetic phosphodiesterase inhibitor, IBMX, at a concentration of 0.2mM. This agent caused a significant ($P<0.01$) elevation of cAMP content, with a temporal profile which closely resembled the changes expressed by caput spermatozoa incubated at a density of $20x10^6$/ml (Fig 13).

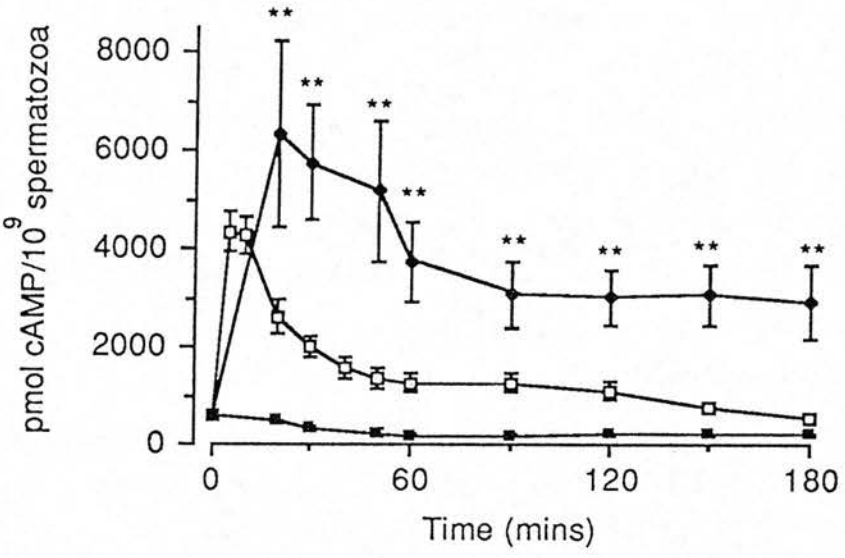



Figure 13. Cyclic AMP content (picomoles cAMP per $10^9$ spermatozoa) expressed by spermatozoa from the caput epididymis of the hamster incubated for a 3h period under the following conditions:

Open squares - At a density of $20x10^6$/ml in medium MT-1,

Closed squares - At a density of $1x10^6$/ml in medium MT-1,

Closed diamonds - At a density of $1x10^6$/ml in medium MT-1 plus 0.2mM IBMX. Significance levels are: **, $P<0.01$. Values shown are mean ± standard error, n=6.

## D. Discussion.

Spermatozoa from the bull and ram exhibit an increase in cAMP content as they mature in the epididymis (Hoskins et al, 1974; Amann et al, 1982), and non-progressive movement can be induced in immature spermatozoa from the bull and hamster by artificially elevating their cAMP content using phosphodiesterase inhibitors (Hoskins et al, 1975b; Kann and Serres, 1980). In view of this, it has been postulated that the acquisition of the potential for movement which occurs during epididymal maturation is related to an increase in cAMP content (Garbers and Kopf, 1980; Tash and Means, 1983). However, Del Rio and Raisman (1978) reported that the cAMP levels of rat spermatozoa decreased during epididymal transit. This study has confirmed and extended this observation by showing that immotile spermatozoa from the caput epididymis of both the rat and the hamster contain significantly higher levels of cAMP than spermatozoa which have completed epididymal maturation, both in undiluted form and upon release into media at a density adjusted to $20x10^6$/ml. As the induction of coordinated flagellar movement in a number of different motile systems has been shown to require the cAMP-dependant phosphorylation of a 56kd axonemal protein named axokinin (Tash, et al, 1984), these findings indicate that the inability of caput spermatozoa from these species to express mature motility patterns must be due to some block downstream from the requirement for cAMP.

Spermatozoa from both the caput and cauda epididymis of the hamster responded to release into medium MT-1 at an adjusted density of $20x10^6$/ml with a sharp rise in cAMP content over basal, undiluted levels. Rat spermatozoa from both these epididymal regions responded to dilution at this density in a similar manner. Increases in the cAMP content of caudal spermatozoa in response to dilution have previously been reported for the hamster (Morton et al, 1974), bull (Cascieri et al, 1976), guinea pig (Hyne and Garbers, 1979a) and mouse (Stein and Fraser, 1984).

In contrast to the situation seen when spermatozoa are diluted to a

cell concentration of $20x10^6$/ml, a rapid increase in cAMP levels is not seen when incubations are performed at the lower cell density of $1x10^6$/ml. It would therefore appear that the initiation of caudal sperm motility which occurs upon dilution does not involve an increase in cAMP levels as has been previously suggested for the hamster (Morton et al, 1974), bull (Cascieri et al, 1976), boar (Okamura and Sugita, 1983), and mouse (Okumura et al, 1985).

Incubation of caput spermatozoa at $1x10^6$/ml in the supernatant of incubations performed at $20x10^6$/ml caused a significant ($P<0.01$) elevation of cAMP content to levels comparable with those seen upon dilution at the higher density. This suggests that the stimulation of cAMP levels seen upon dilution to $20x10^6$/ml involves the mediation of a factor present in epididymal plasma. As inhibitors of phosphodiesterase activity are known to be present in the epididymis of the rat (Benau et al, 1986), the effects of incubating caput spermatozoa at a density of $1x10^6$/ml in the presence of the phosphodiesterase inhibitor IBMX were determined. This agent elevated cAMP to levels similar to, though significantly ($P<0.01$) greater than those shown by caput sperm incubated at $20x10^6$/ml in MT-1. These data are therefore consistent with the hypothesis that the difference in cAMP levels between incubations performed at $20x10^6$/ml and $1x10^6$/ml is due to the dilution of a phosphodiesterase inhibitor present in epididymal plasma.

Closer analysis of previous studies which have reported increases in cAMP content upon dilution revealed that those performed on the hamster, bull and mouse (Morton et al, 1974; Cascieri et al, 1976; Stein and Fraser, 1984) had made no allowance for epididymal fluid contamination. Thus the increases in cAMP content observed to accompany dilution in these species may result from the action of some exogenous agent in these incubations. The study which utilised the guinea pig as an animal model (Hyne and Garbers, 1979a) did wash the spermatozoa once prior to incubation. However, the relatively high cell concentration at which subsequent incubations were performed may have potentiated the action of any

remaining plasma factors.

Although Carr and Acott (1984) showed that the cAMP content of caudal epididymal spermatozoa from the bull did not increase upon dilution, eventhough epididymal plasma was not removed prior to incubation, the buffer utilised by this group did not contain calcium, which has been shown to be required for the expression of the increase in cAMP content (see below).

The rise in cAMP levels which accompanied dilution of both caput and caudal spermatozoa from the hamster at $20x10^6$/ml was severely inhibited by the removal of exogenously added calcium from the medium. As the cAMP content of spermatozoa incubated in NAC in the presence of the phosphodiesterase inhibitor IBMX is significantly ($P<0.01$) lower than the identically treated spermatozoa in MT-1, it would appear that the synthesis of cAMP has an obligatory requirement for this cation. A similar calcium dependancy has been shown to exist in guinea pig caudal spermatozoa (Hyne and Garbers, 1979a).

This group further reported that treatment of these spermatozoa with the calmodulin antagonist trifluoperazine inhibited the calcium dependant rise in cAMP levels associated with dilution. Extensions of their studies led them to conclude that the sperm adenylate cyclase of this species is calmodulin dependant, as 100μM trifluoperazine inhibited the adenylate cyclase activity of caudal sperm homogenates by 50% (Hyne and Garbers, 1979b). However, the dose of this reagent utilised is far in excess of the concentration necessary to neutralise the activity of calmodulin dependant enzymes such as the $Ca^{2+}$-transport ATPase or brain phosphodiesterase (Gietzen et al, 1982). Under these circumstances, the inhibitory action of this lipophilic compound probably results from its ability to induce non-specific perturbance of the plasma membrane, rather than a specific disruption of a calmodulin dependant process. To determine whether the rise in cAMP levels depended upon the action of calmodulin, both caput and caudal hamster spermatozoa at a density of $20x10^6$/ml in media MT-1, were

challenged with calmidazolium (compound R 24571), a more efficient and specific calmodulin inhibitor than trifluoperazine, at a dose (0.5μM) sufficient to inhibit calmodulin-dependant enzyme activity (Gietzen et al, 1982). No diminution in the cAMP content of either cell type was observed, either when spermatozoa were simultaneously exposed to both calmidazolium and calcium, or when caudal cells were preincubated in the presence of calmidazolium and the absence of calcium. These results indicate a lack of involvement for this calcium dependant modulator protein in cAMP synthesis in this species. That a slight elevation of cAMP content resulted from treatment with this reagent may indicate that the phosphodiesterase of these cells is calmodulin dependant, as has been shown for rat spermatozoa (Wasco and Orr, 1984).

As changes in membrane phospholipid content are known to affect adenylate cyclase activity (Houslay et al, 1976; Bakardjiera et al, 1979; Salesse and Garnier, 1984), and as hamster spermatozoa are known to possess phospholipase A2 activity (Llanos et al, 1982), the effects of the phospholipase A2 inhibitor, -p-dibromoacetophenone on the increase in cAMP levels were determined. This agent had no significant effect on the cAMP content of either cell type.

In conclusion, these experiments have shown that no direct relationship exists between the cAMP content of hamster and rat spermatozoa and their potential for motility. Also, although spermatozoa incubated at a concentration of $20x10^6$/ml show a rapid calcium, but not calmodulin, dependant increase in cAMP levels upon dilution, this rise does not appear to be involved in the initiation of motility upon dilution, as it is absent in incubations performed at a density of $1x10^6$/ml. The elevation of cAMP content seen at the higher density may be due to a phosphodiesterase inhibitor present in epididymal plasma.

# Chapter 3. The relationship between cAMP levels and the expression of hyperactivated motility.

## A. Introduction.

Mammalian spermatozoa released at ejaculation require a period of capacitation within the female genital tract before penetration of the oocyte vestments and fertilization can be achieved (Chang, 1951; Austin, 1952). Spermatozoa of many different species are known to express altered patterns of motility concomitant with the terminal stages of this process (See general introduction; Burkman, 1984; Boatmen and Bavister, 1984). These changes, termed hyperactivation (Yanagimachi, 1981), are characterized by vigorous, large amplitude, whiplash-like flagellar beats, with the sperm heads tracing erratic trajectories (Katz and Yanagimachi, 1980). Such modifications are thought to facilitate the passage of spermatozoa through both the cumulus oophorus and the zona pellucida (Katz et al, 1986).

The exact biochemical mechanisms underlying this modulation of sperm movement remain unknown. However, the expression of hyperactivated motility is known to depend upon the presence of calcium in the external medium (Yanagimachi and Usui, 1974; Fraser, 1977; Shams-Borhan and Harrison, 1981; Yanagimachi, 1982). Indirect evidence further suggests that capacitation in vitro is accompanied by a net uptake of calcium (Singh et al, 1978; Triana et al, 1980; Singh et al, 1980). The adenylate cyclase enzyme, which is known to be calcium (Kopf and Vacquier, 1985) and possibly calmodulin (Hyne and Garbers, 1979a, b; Gordeladze et al, 1982) dependant in spermatozoa, has also been shown to increase in activity during capacitation (Morton and Albagli, 1973; Stein and Fraser, 1984). These factors suggest that a calcium-dependant increase in the intracellular levels of cyclic 3',5'-adenosine monophosphate (cAMP) should occur during capacitation. Indeed, artificial elevation of sperm cAMP content following treatment with membrane permeant analogues of cAMP or phosphodiesterase inhibitors, has been found to induce hyperactivated motility in rhesus monkey spermatozoa (Boatman and Bavister, 1984), stimulate its incidence in both hamster (Mrsny and Meizel, 1980) and human spermatozoa (Burkman, 1984), and accelerate its onset in spermatozoa from

the mouse (Fraser, 1979; 1981), although a subsequent study (Cooper, 1984) has failed to confirm the latter observation.

However, until recently, direct measurements of the cAMP content of capacitating spermatozoa have failed to relate changes in the level of this nucleotide to the onset and expression of hyperactivated motility (Hyne and Garbers, 1979a; Stein and Fraser, 1984). In the previous chapter it was shown that caudal spermatozoa from the hamster, incubated at a density of $1x10^6$/ml, exhibit a progressive increase in cAMP content which caput spermatozoa incubated under identical conditions are unable to express. The aim of the experiments detailed below was to determine whether this increase in cAMP content is related to the expression of hyperactivated motility during capacitation.

## B. Materials and Methods.

Both the media employed in this study and the techniques utilised for; isolation of spermatozoa, extraction of cAMP, determination of cAMP levels and statistics are as outlined in chapter 2.

i. Motility and acrosomal status assessment. Percentage motility and the percentage of motile cells showing hyperactivated motility were estimated using a microscope with a heated stage set at 37°C. At least 100 spermatozoa were counted for each separate determination with the aid of an eyepiece graticule. These spermatozoa were also scored for the presence or absence of acrosomal caps using phase contrast optics.

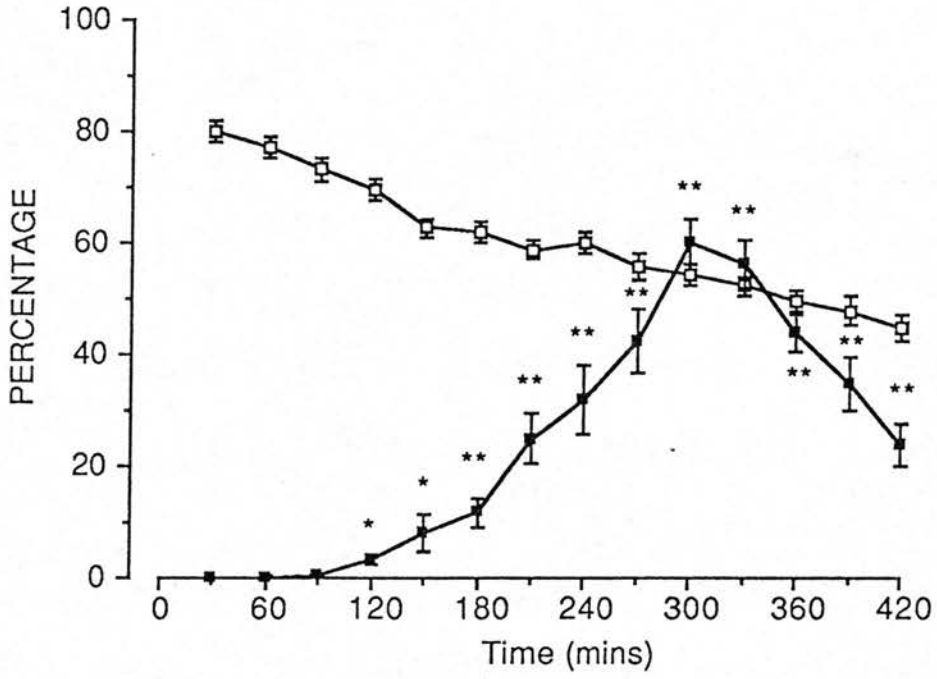



Figure 14. Percentage motility (open squares) and percentage hyperactivated motility (closed squares) expressed by spermatozoa from the hamster cauda epididymis incubated for a 7h period in medium MT-1 at a density of $1x10^6$/ml. Significance levels are: *, $P<0.05$; **, $P<0.01$. Values shown are mean ± standard error, n=6.

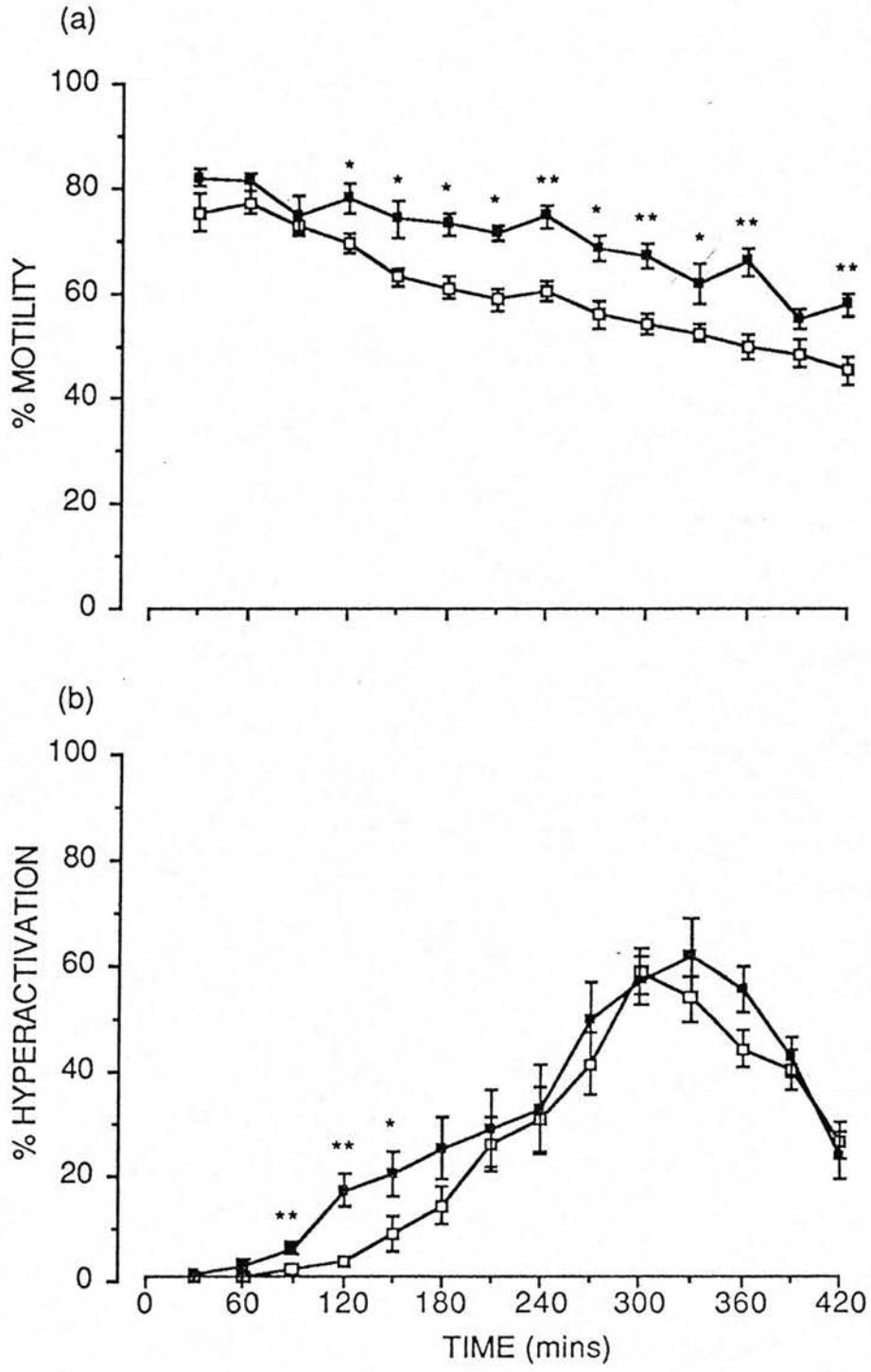



Figure 15. Percentage motility (a) and percentage hyperactivated motility (b) expressed by spermatozoa from the hamster cauda epididymis incubated at a density of $1x10^6$/ml for a 7h period in either medium MT-1 (open squares), or medium MT-1 plus 0.2mM IBMX (closed squares). Significance levels are: *, $P<0.05$; **, $P<0.01$. Values shown are mean ± standard error, n=6.

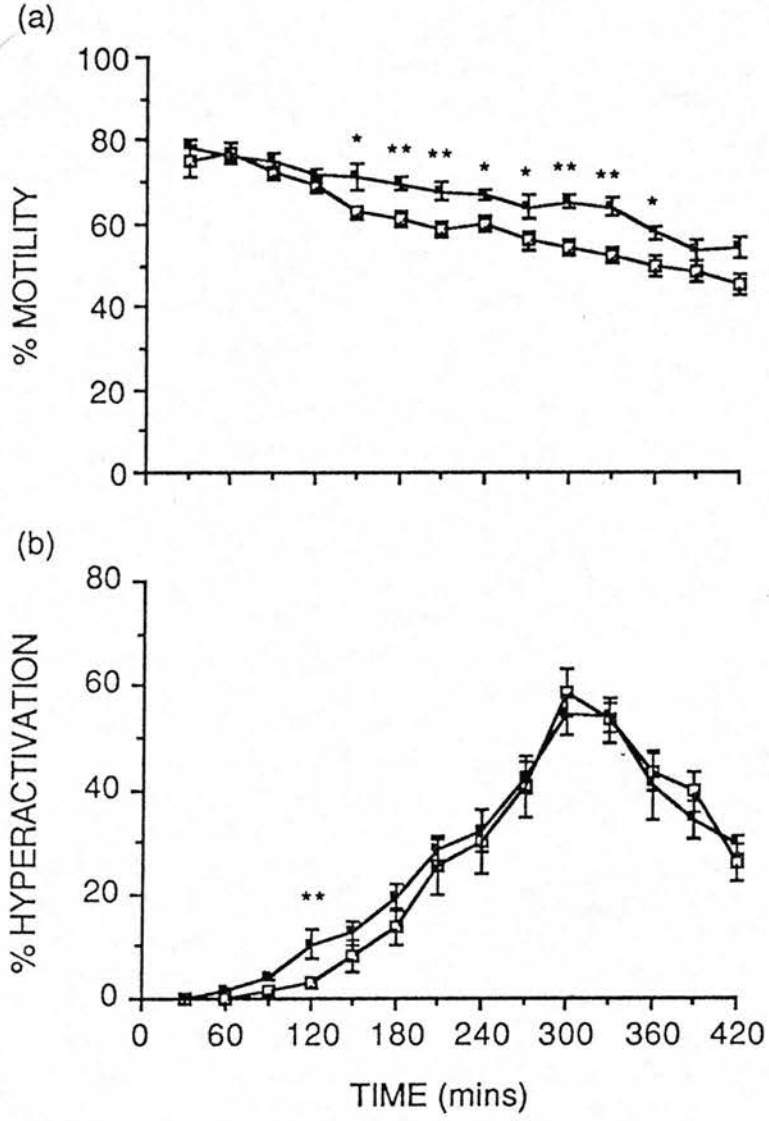



Figure 16. Percentage motility (a) and percentage hyperactivated motility (b) expressed by spermatozoa from the hamster cauda epididymis incubated at a density of $1x10^6$/ml for a 7h period in either medium MT-1 (open squares), or medium MT-1 plus 0.5mM dibutryl cAMP (closed squares). Significance levels are: *, $P<0.05$; **, $P<0.01$. Values shown are mean ± standard error, n=6.

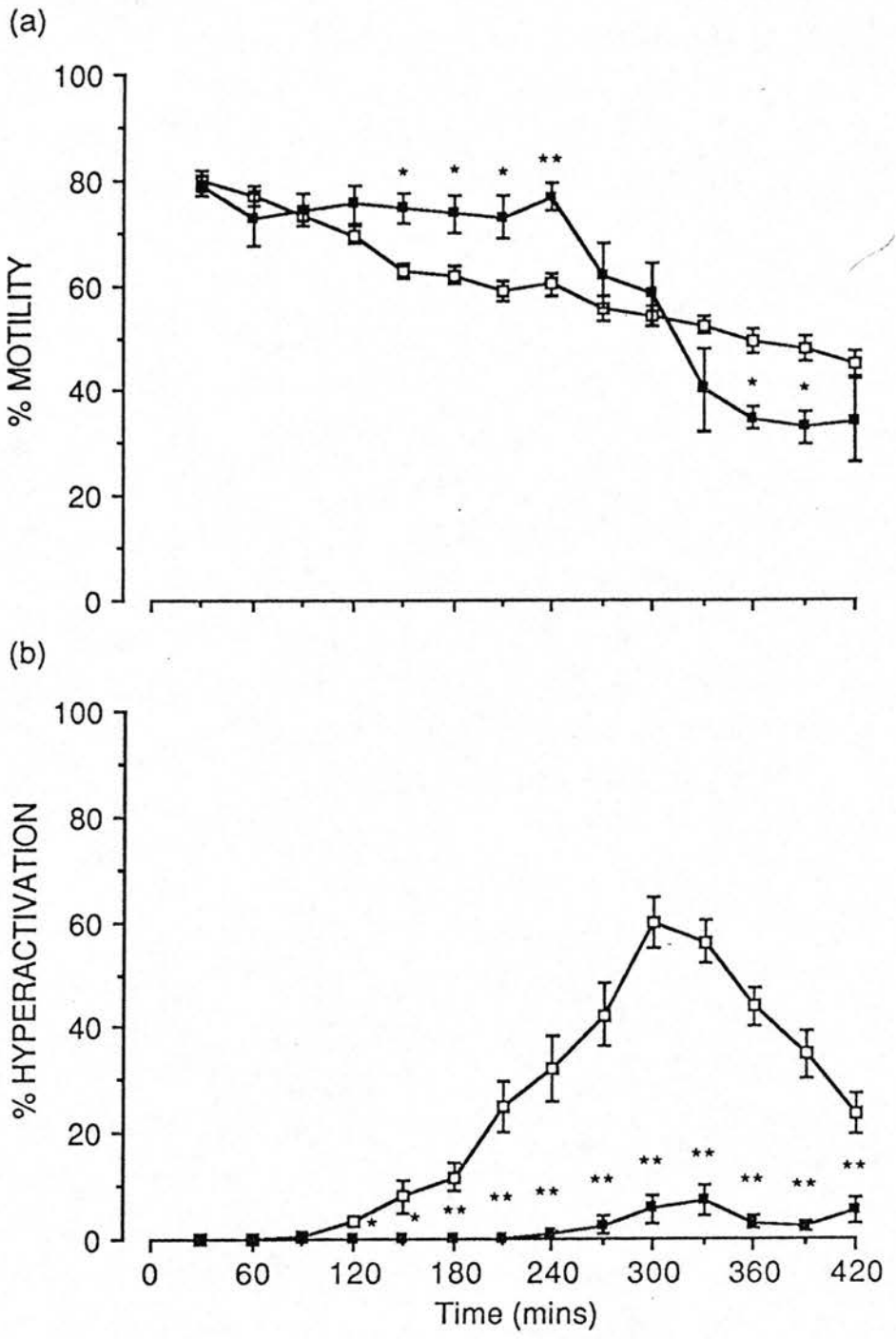



Figure 17. Percentage motility (a) and percentage hyperactivated motility (b) expressed by spermatozoa from the hamster cauda epididymis incubated at a density of 1x10$^6$/ml for a 7h period in either medium MT-1 (open squares), or medium NAC (closed squares). Significance levels are: *, $P<0.05$; **, $P<0.01$. Values shown are mean ± standard error, n=6.

## C. Results.

i. Motility analysis - 1x10^6/ml.

a. In complete medium. After 30 min of incubation in medium MT-1, 75% of caudal spermatozoa were motile. Percentage motility then slowly declined with time, reaching values of 61, 54 and 45 after 3, 5 and 7h respectively (Fig 14). Significant ($P<0.05$) levels of hyperactivation were first seen after two hours of incubation. Thereafter, percentage hyperactivated motility steadily increased, reaching a maximum 5h after initial dilution, at which time 58% of live spermatozoa expressed this pattern of motility.

Addition of 0.2mM of the phosphodiesterase inhibitor isobutyl methyl xanthine (IBMX) to the incubations performed in MT-1 significantly ($P<0.01$) stimulated the percentage motility (Fig 15a), and significantly ($P<0.01$) advanced the onset of hyperactivated motility (Fig 15b).

Addition of 0.5mM of the membrane permeant analogue of cAMP, dibutryl cAMP, to the incubations performed in MT-1 resulted in a significant ($P<0.01$) stimulation of percentage motility between 150 and 360 mins after dilution (Fig 16a). The percentage of spermatozoa exhibiting hyperactivation was also significantly ($P<0.01$) increased after 120 mins of incubation (Fig16b), in a manner similar to, although less pronounced than that produced by IBMX (see above).

b. In medium NAC. Incubation of caudal spermatozoa at a density of $1x10^6$/ml in medium MT-1 devoid of exogenously added calcium (NAC) caused a significant ($P<0.01$) stimulation of percentage motility over the first half of the incubation period, but levels subsequently fell to become significantly ($P<0.01$) lower than those expressed in complete MT-1 after 6h of incubation (Fig 17a). Spermatozoa incubated in this medium, however, exhibited a drastic reduction in percentage hyperactivation, significantly ($P<0.01$) lower than the levels expressed in MT-1 (Fig 17b). In medium NAC, hyperactivated motility was never expressed by more than 10% of the total

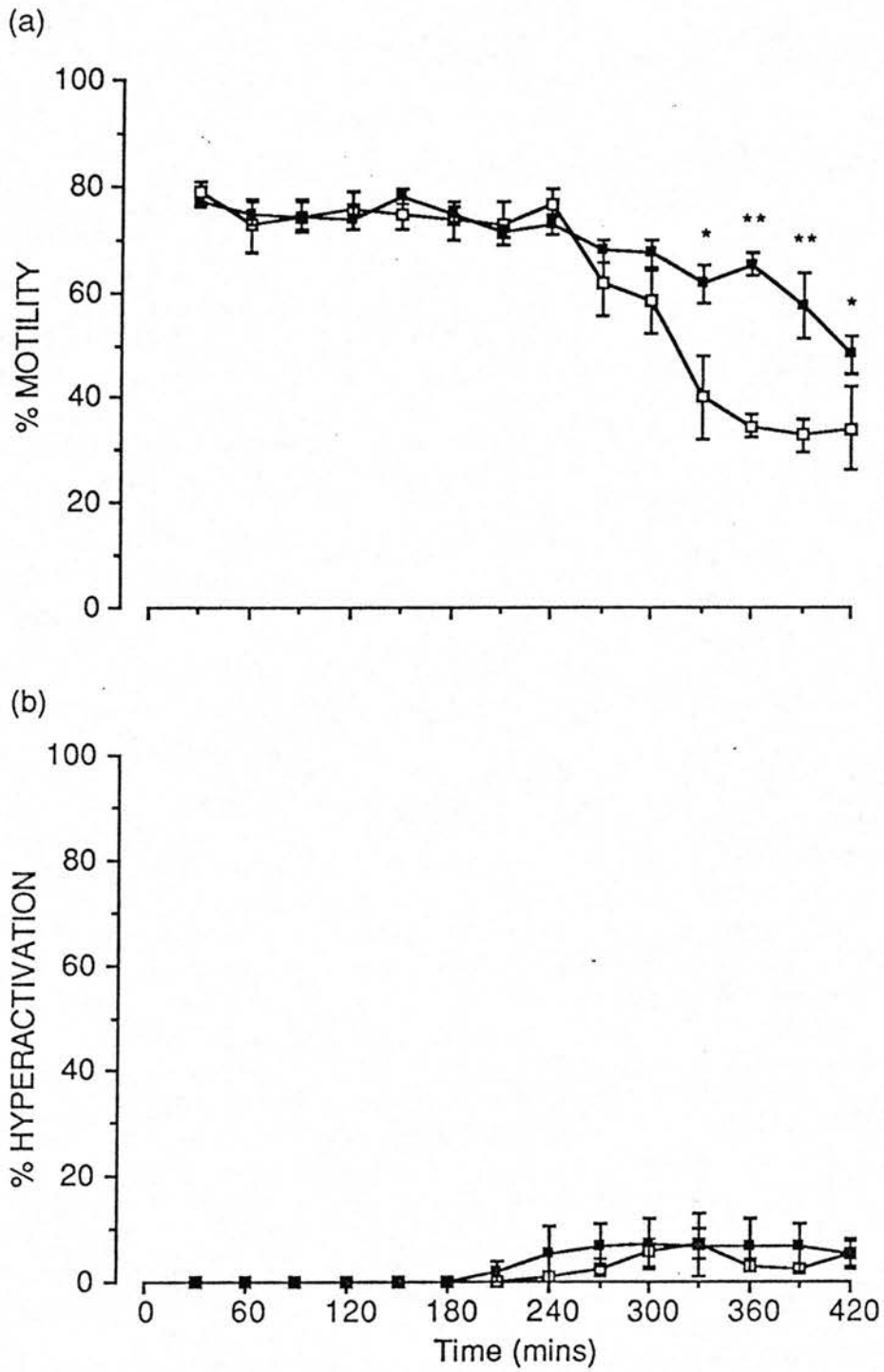



Figure 18. Percentage motility (a) and percentage hyperactivated motility (b) expressed by spermatozoa from the hamster cauda epididymis incubated at a density of $1x10^6$/ml for a 7h period in either medium NAC (open squares), or medium NAC plus 0.2mM IBMX (closed squares). Significance levels are: *, $P<0.05$; **, $P<0.01$. Values shown are mean ± standard error, n=6.

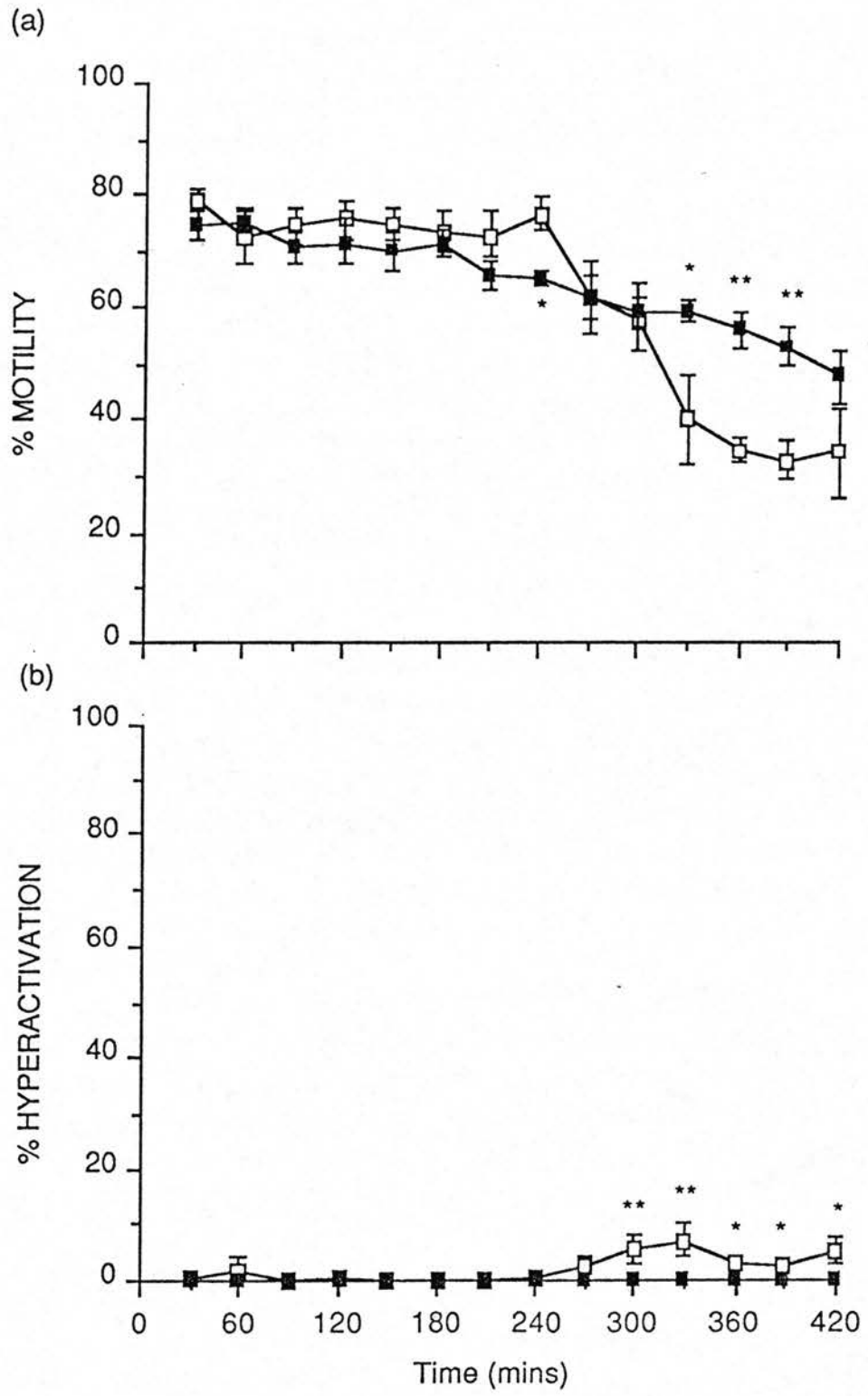



Figure 19. Percentage motility (a) and percentage hyperactivated motility (b) expressed by spermatozoa from the hamster cauda epididymis incubated at a density of $1x10^6$/ml for a 7h period in either medium NAC (open squares), or medium NAC plus 0.5mM dibutryl cAMP (closed squares). Significance levels are: *, $P<0.05$; **, $P<0.01$. Values shown are mean ± standard error, n=6

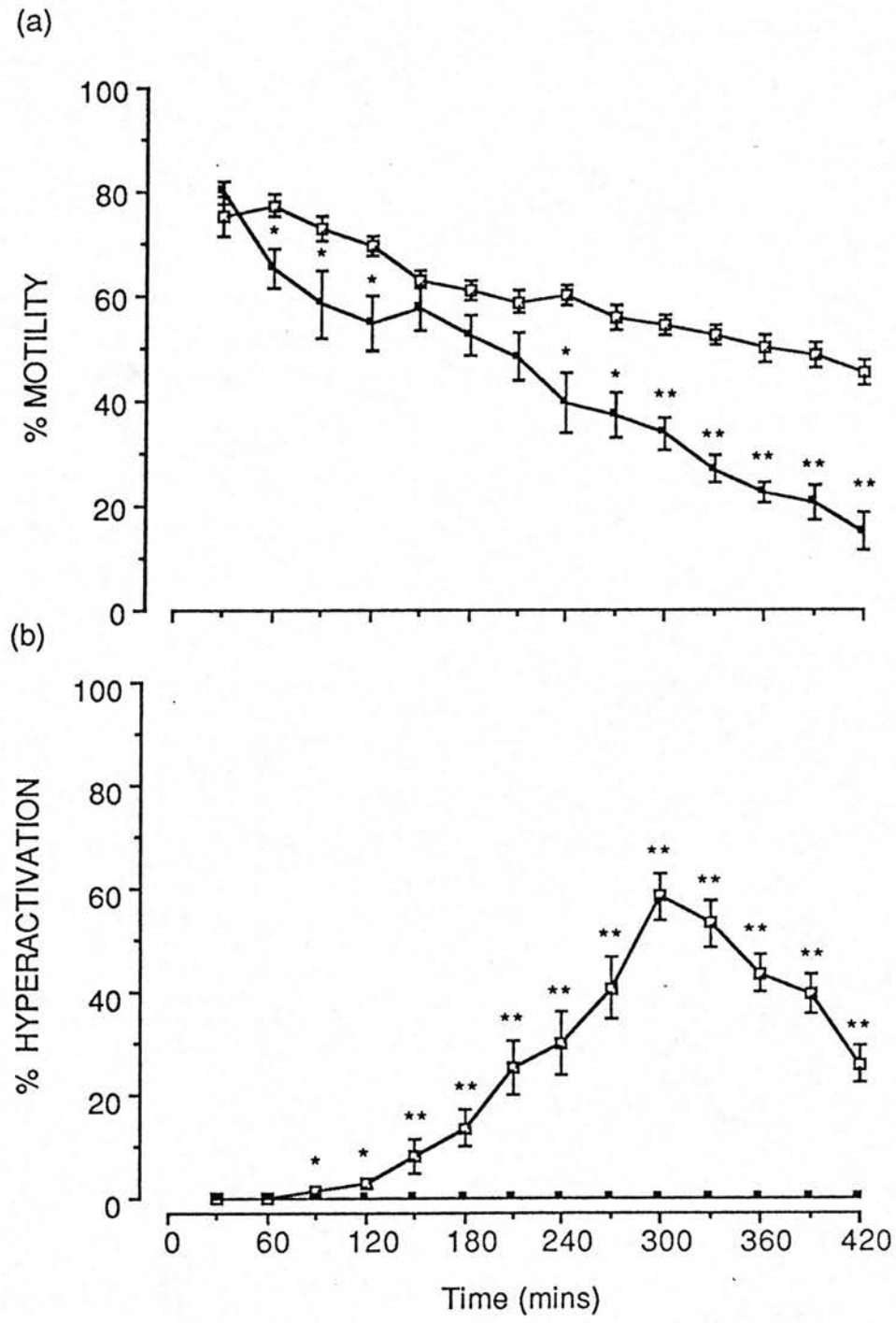



Figure 20. Percentage motility (a) and percentage hyperactivated motility (b) expressed by spermatozoa from the hamster cauda epididymis incubated at a density of $1x10^6$/ml for a 7h period in either medium MT-1 (open squares), or medium MT-1 plus 0.5μM calmidazolium (closed squares). Significance levels are: *, $P<0.05$; **, $P<0.01$. Values shown are mean ± standard error, n=6

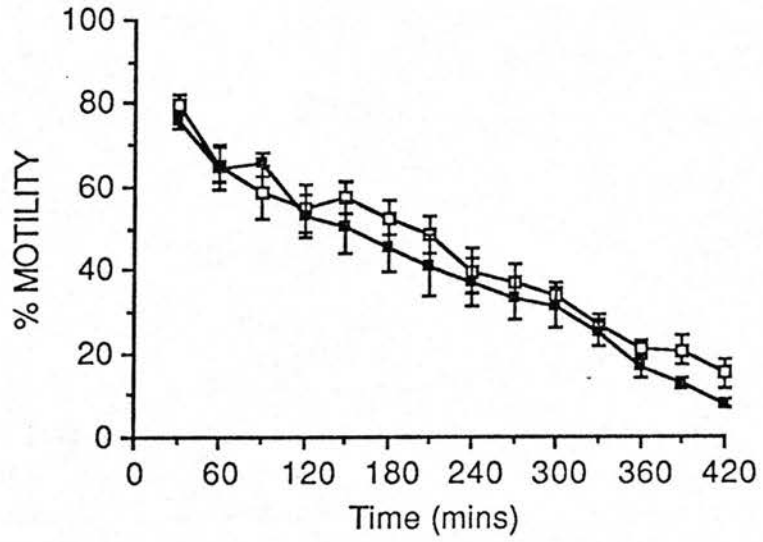



Figure 21. Percentage motility expressed by spermatozoa from the hamster cauda epididymis incubated at a density of $1x10^6$/ml for a 7h period in either medium MT-1 plus 0.5µM calmidazolium (open squares), or medium MT-1 plus both 0.5µM calmidazolium and 0.2mM IBMX (closed squares). Values shown are mean ± standard error, n=6

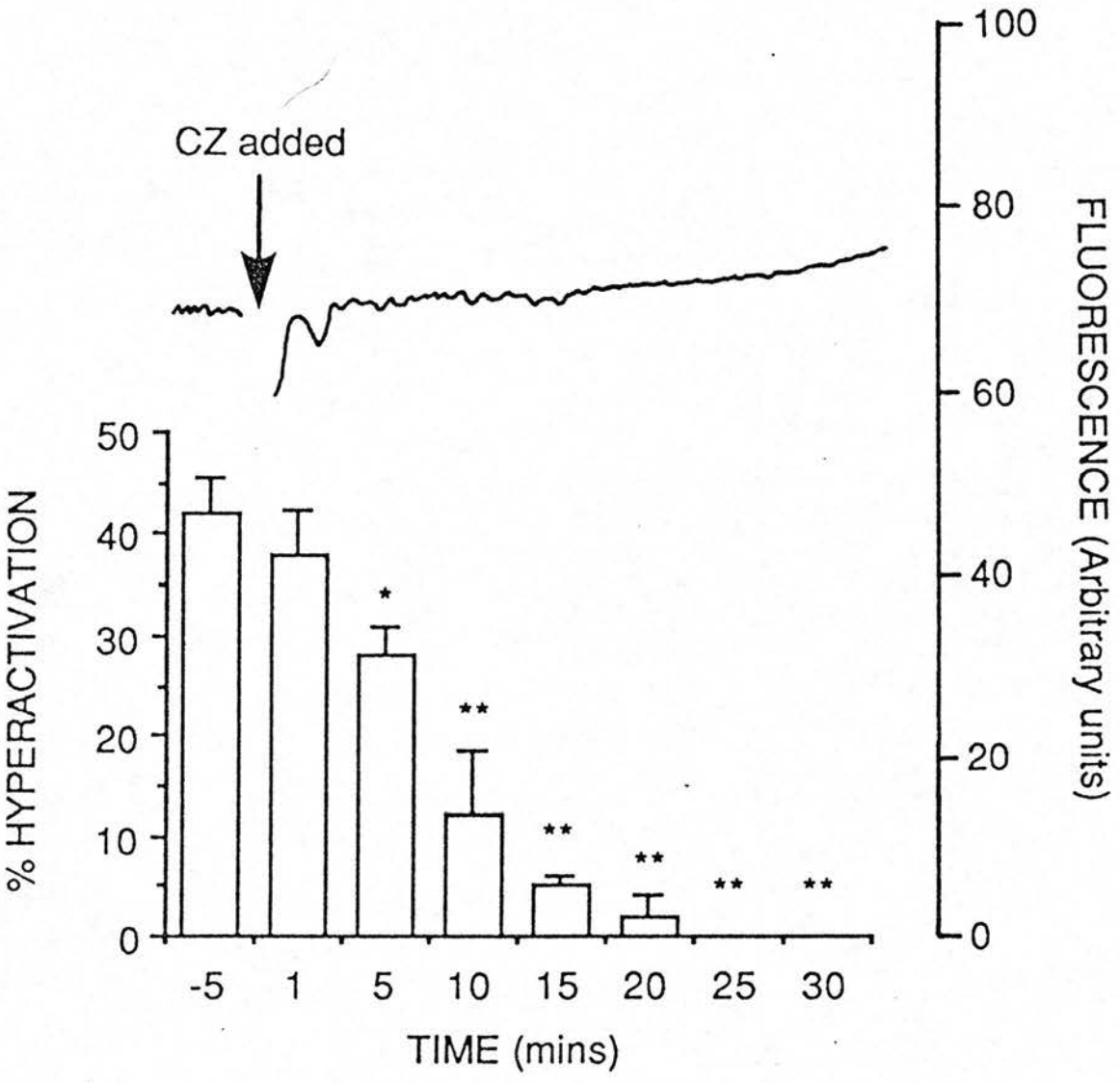



Figure 22. Percentage hyperactivated motility (closed bars) and a representative fluoresence emission trace performed at 492nm (line graph) for spermatozoa from the hamster cauda epididymis pre-incubated for 3h at a density of $1x10^6$/ml in medium MT-1, then treated with 0.5µM calmidazolium. Significance levels are: **, $P<0.01$. Values shown are mean ± standard error, n=6

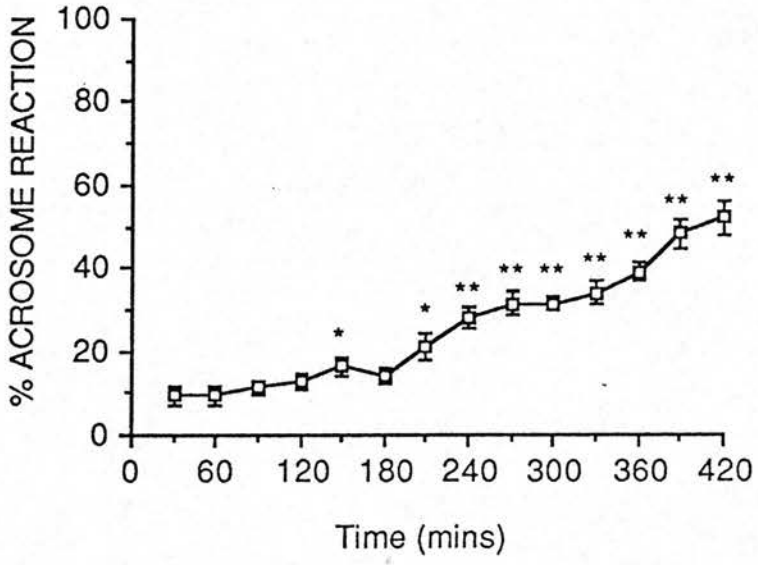



Figure 23. Percentage acrosome reaction determined for spermatozoa from the hamster cauda epididymis incubated for 7h at a density of $1x10^6$/ml in medium MT-1. Significance levels are: * $P<0.05$; **, $P<0.01$. Values shown are mean ± standard error, n=6

motile population, in comparison to peak levels of 58% in MT-1.

Addition of 0.2mM of the phosphodiesterase inhibitor isobutyl methyl xanthine (IBMX) to the incubations performed in NAC significantly ($P<0.01$) stimulated the percentage motility (Fig 18a), but caused only a slight, insignificant increase in percentage hyperactivated motility (Fig 18b).

Addition of 0.5mM dibutryl cAMP to the incubations performed in NAC also significantly ($P<0.01$) stimulated the percentage motility (Fig 19a), but hyperactivation was never expressed in the presence of this reagent (19b), the levels of hyperactivation actually being significantly ($P<0.01$) lower than in medium NAC alone.

c. In the presence of calmodulin inhibitor. Addition of the calmodulin antagonist calmidazolium (compound R24571) at a concentration of 0.5μM (Gietzen et al, 1982) to incubations performed in MT-1 at a density of $1x10^6$/ml caused severe disruption of coordinated motility, with levels being significantly ($P<0.01$) lower than in MT-1 alone (Fig 20a). Hyperactivated motility was never expressed in the presence of this reagent (Fig 20b). Addition of IBMX to these incubations did not alleviate the effects of the calmodulin antagonist on motility (Fig 21), despite a significant elevation of intracellular cAMP levels (see figure 30).

Spermatozoa pre-loaded with Quin-2 (for loading protocol, see materials and methods, chapter 4) showed no alteration in their fluorescence emission either immediatley upon the addition of 0.5μM calmidazolium or over the period of subsequent motility disruption (Fig 22).

ii. Acrosomal status. After 30 mins of incubation in medium MT-1, 9% of caudal spermatozoa had lost their acrosomal caps. It was assumed that this figure represented a background acrosomal loss secondary to a reduction in cell viability, and not a physiological acrosome reaction. Levels of acrosome reacted spermatozoa rose significantly ($P<0.05$) above this basal level after 150 mins of incubation, when levels reached a value of 16% (Fig 23). Thereafter, the size of the acrosome reacted population

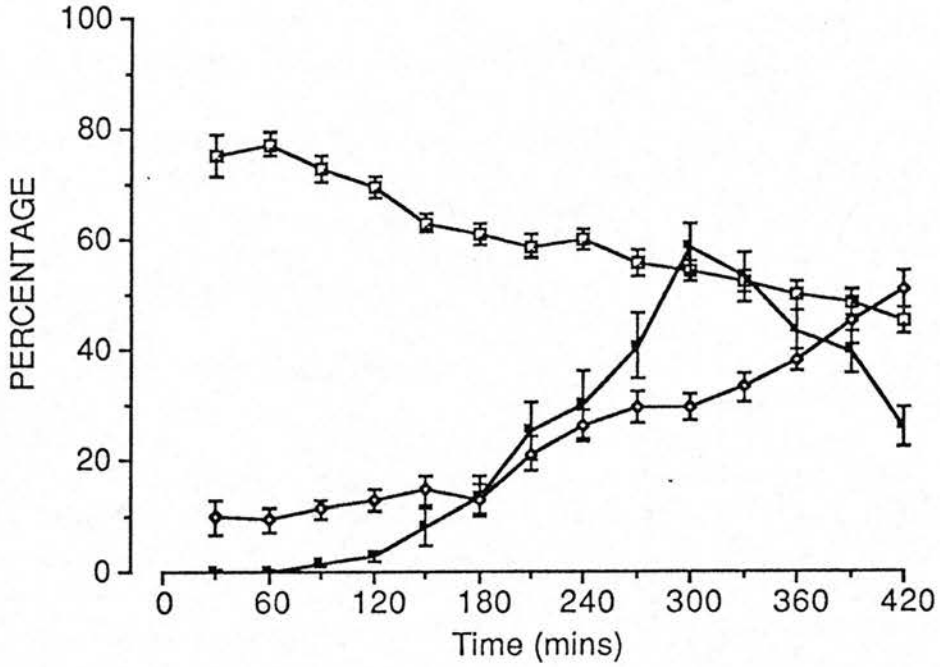



Figure 24. Percentage motility (open squares), percentage hyperactivated motility (Closed squares) and percentage acrosome reaction (open diamonds) determined for spermatozoa from the hamster cauda epididymis incubated for 7h at a density of $1x10^6$/ml in medium MT-1. Values shown are mean ± standard error, n=6

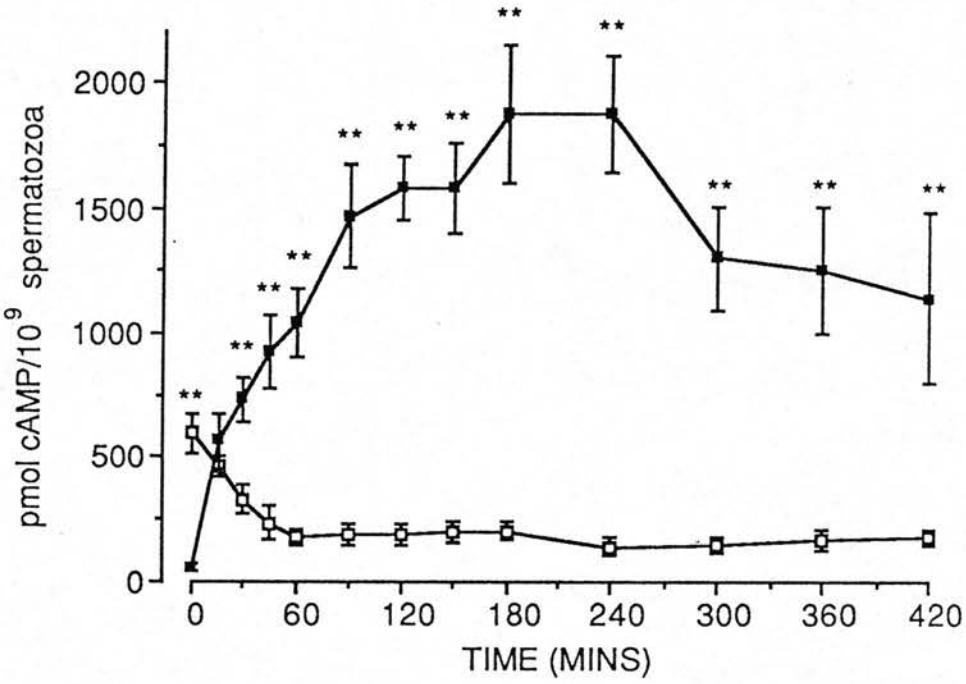



Figure25. Cyclic AMP content (picomoles cAMP per $10^9$ spermatozoa) expressed by spermatozoa from the hamster caput (open squares) or cauda (closed squares) epididymis, incubated at a density of $1x10^6$/ml over a 7h period in medium MT-1. Significance levels are: *, $P<0.05$; **, $P<0.01$. Values shown are mean ± standard error, n=6.

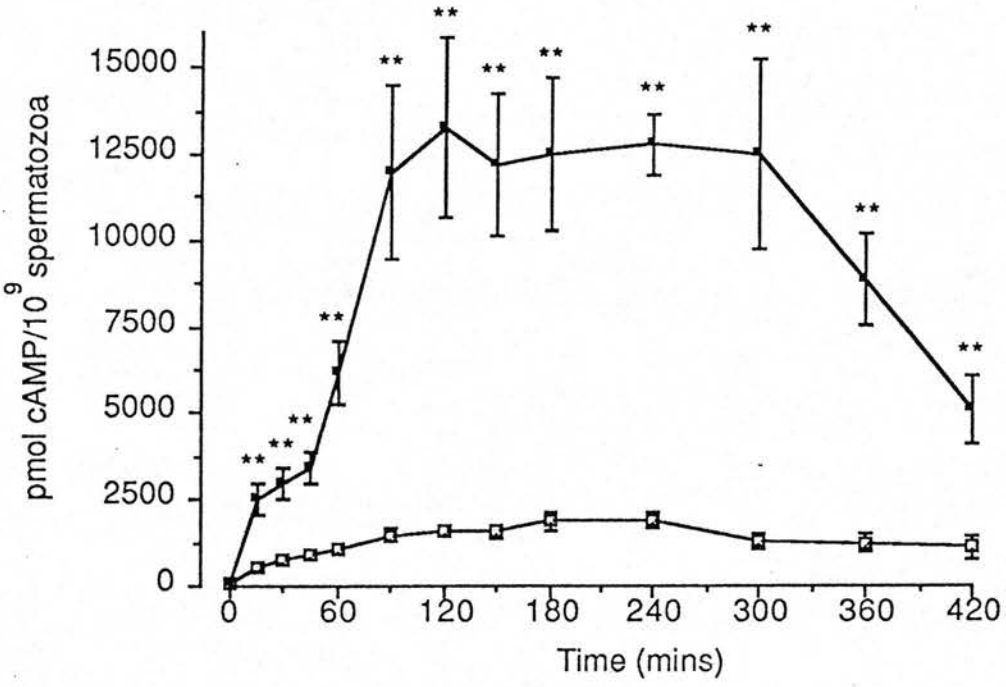



Figure 26. Cyclic AMP content (picomoles cAMP per $10^9$ spermatozoa) expressed by spermatozoa from the hamster cauda epididymis, incubated at a density of $1x10^6$/ml over a 7h period in either medium MT-1 (open squares), or medium MT-1 plus 0.2mM IBMX .(closed squares). Significance levels are: **, $P<0.01$. Values shown are mean ± standard error, n=6.

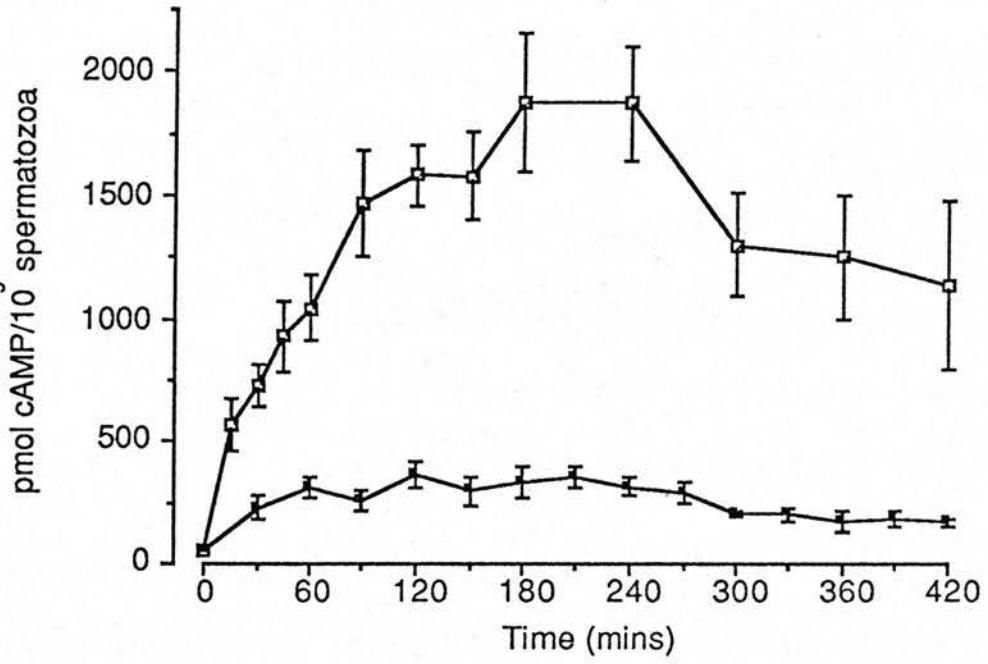



Figure27. Cyclic AMP content (picomoles cAMP per $10^9$ spermatozoa) expressed by spermatozoa from the hamster cauda epididymis, incubated at a density of $1x10^6$/ml over a 7h period in either medium MT-1 (open squares), or medium NAC (closed squares). Values differ significantly ($P<0.01$) between the two treatment groups. Values shown are mean ± standard error, n=6.

increased progressively, to reach levels of 31 and 52 % after 5 and 7 hours respectively. It would thus appear that capacitating hamster spermatozoa exhibit peak levels of hyperactivated motility prior to the maximal attainment of the acrosome reaction (Fig 24).

### iii Cyclic AMP levels - response to dilution at $1x10^6$/ml.

a. In complete medium. In the chapter 2 it was shown that caudal spermatozoa incubated for a 3h period at a density of $1x10^6$/ml exhibited a progressive increase in cAMP content which spermatozoa from the caput epididymis were unable to express. By extending the incubation period to 7h, the cAMP content of caudal spermatozoa was shown to reach a plateau of around 1875 pmols cAMP/$10^9$ spermatozoa between 3 and 4 h after initial dilution (Fig 25). This rise in cAMP levels thus precedes the expression of hyperactivated motility by these cells. After this time, cAMP levels decreased slightly, although they remained significantly ($P<0.01$) elevated over $T_o$ values after 7h of incubation. Caput cAMP content remained low throughout this extended incubation period.

Incubation of spermatozoa in MT-1 plus IBMX (0.2mM), conditions which significantly accelerated the onset of hyperactivated motility (Fig 15b), caused a dramatic increase in cAMP content, with levels being significantly ($P<0.01$) higher than those expressed in MT-1 alone within 15min of dilution (Fig 26).

b. In medium NAC. The supression of hyperactivated motility which resulted from incubation in medium NAC was associated with a severely attenuated rise in cAMP content, with levels never exceeding 400 pmol cAMP/$10^9$ spermatozoa (Fig 27). However, dilution in NAC was not completely without effect, as a significant ($P<0.01$) increase in sperm cAMP content over the basal levels was evoked, with levels reaching 228 pmol cAMP/$10^9$ spermatozoa after 30 min incubation, compared to 56 pmol

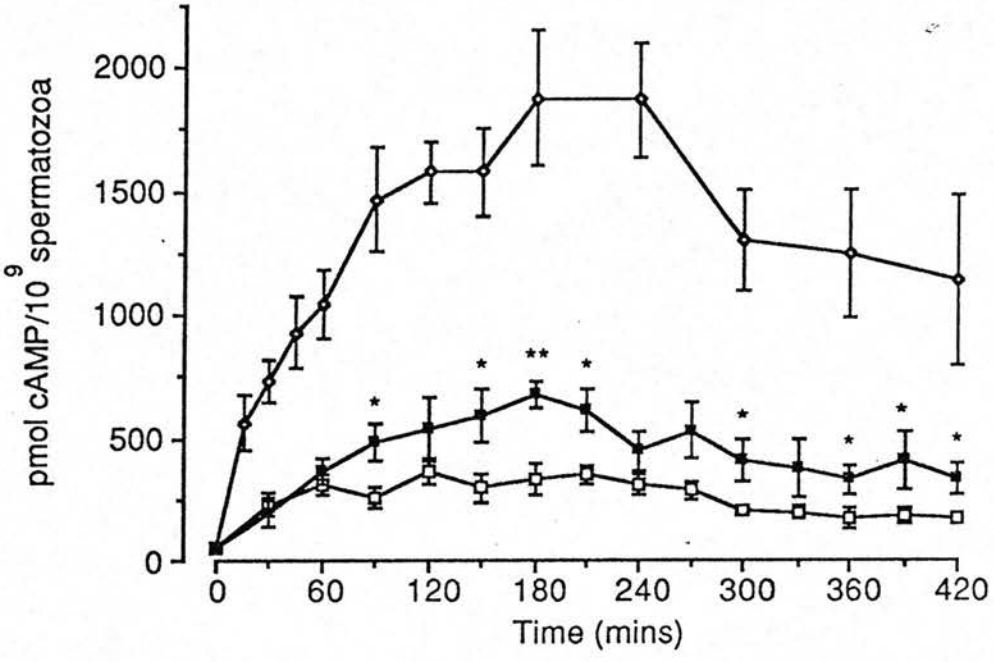



Figure 28. Cyclic AMP content (picomoles cAMP per $10^9$ spermatozoa) expressed by spermatozoa from the hamster cauda epididymis, incubated at a density of $1x10^6$/ml over a 7h period in; medium MT-1 (open diamonds), medium NAC (open squares), or medium NAC plus 0.2mM IBMX (closed squares). Significance levels are: *, $P<0.05$; **, $P<0.01$. Values shown are mean ± standard error, n=6.

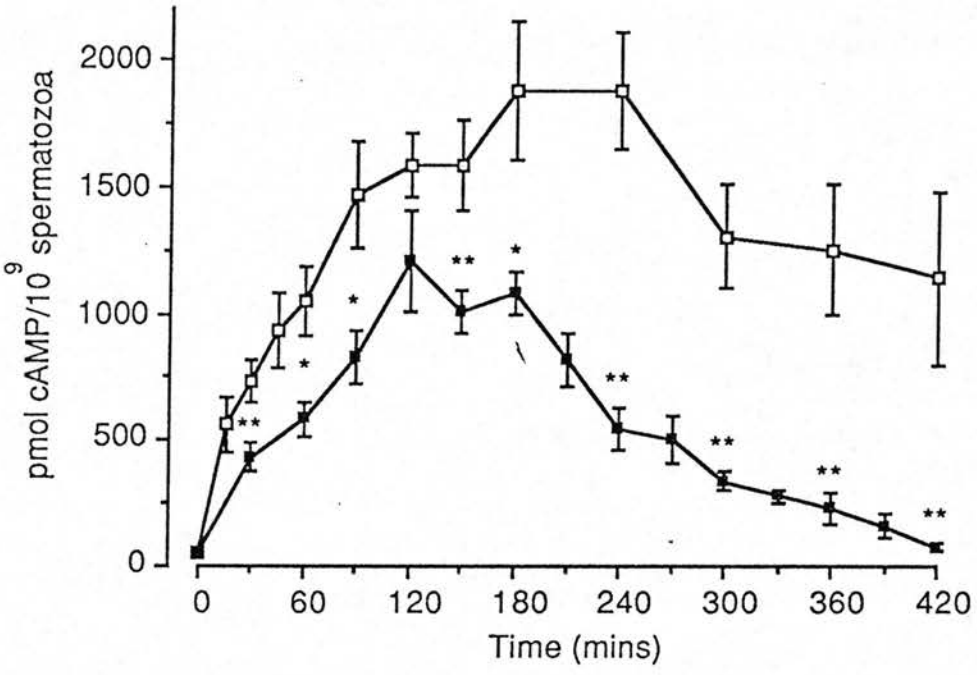



Figure 29. Cyclic AMP content (picomoles cAMP per $10^9$ spermatozoa) expressed by spermatozoa from the hamster cauda epididymis, incubated at a density of $1x10^6$/ml over a 7h period in either medium MT-1 (open squares) or medium MT-1 plus 0.5µM calmidazolium (closed squares). Significance levels are: *, $P<0.05$; **, $P<0.01$. Values shown are mean ± standard error, n=6.

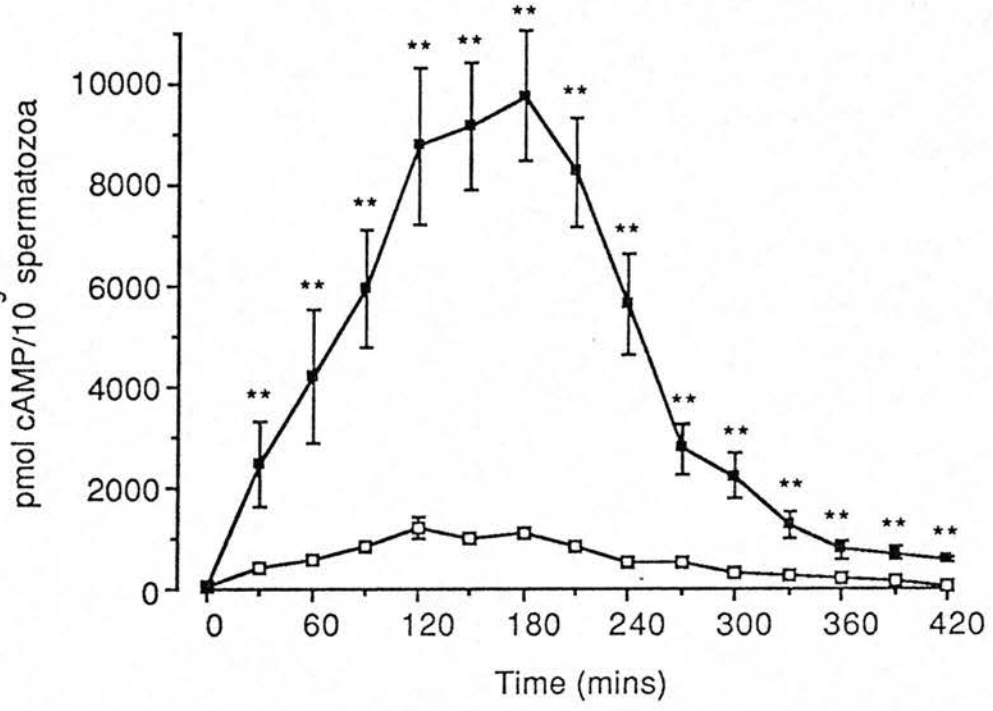



Figure 30. Cyclic AMP content (picomoles cAMP per $10^9$ spermatozoa) expressed by spermatozoa from the hamster cauda epididymis, incubated at a density of $1x10^6$/ml over a 7h period in either medium MT-1 plus 0.5µM calmidazolium (open squares) or medium MT-1 plus both 0.5µM calmidazolium and 0.2mM IBMX (closed squares). Significance levels are: **, $P<0.01$. Values shown are mean ± standard error, n=6.

cAMP/$10^9$ spermatozoa in undiluted cells.

Addition of IBMX (0.2mM) to incubations performed in medium NAC significantly ($P<0.01$) stimulated sperm cAMP content over a time course of 7h (Fig 28). However, these elevated levels were still significantly ($P<0.01$) lower than those expressed in MT-1, and were not associated with a stimulation of hyperactivated motility.

c. In the presence of calmodulin antagonist.. The disruption of hyperactivated motility seen upon addition of calmidazolium to spermatozoa incubated in MT-1 was associated with a significant ($P<0.01$) reduction in cAMP content, with the disparity between treatment and control increasing over the latter part of the incubation (Fig 29). Addition of 0.2mM IBMX to these incubations caused a dramatic elevation of cAMP content to levels significantly ($p<0.01$) higher than those expressed in MT-1 (Fig 30), although the movement characteristics of the spermatozoa remained unchanged (see above).

## D. Discussion

It is widely accepted that increases in the cAMP content of spermatozoa result in a stimulation of motility (see chapter 1; Garbers and Kopf, 1980; Tash and Means, 1983). Indirect evidence from experiments utilising phosphodiesterase inhibitors or membrane permeant analogues of cAMP have also implicated a role for this nucleotide in the expression of hyperactivated motility (Fraser, 1979, 1981; Mrsny and Meizel, 1980; Burkman, 1984; Boatman and Bavister, 1984). However, previous studies which have measured the cAMP content of capacitating spermatozoa have failed to relate changes in the level of this nucleotide to the appearance of hyperactivated motility (Hyne and Garbers, 1979a; Stein and Fraser, 1984).

The experiments detailed above have shown that capacitation of hamster spermatozoa is associated with a progressive rise in cAMP levels, and that this phenomenon precedes the expression of hyperactivation. In turn, spermatozoa appear to display maximal levels of hyperactivation before peak levels of acrosome reaction are attained. The observed timing of these separate events is in agreement with the findings of previous studies (Mahi and Yanagimachi, 1973; Fraser 1977, 1982).

These results also provide confirmation of the results published by other groups who have reported that the expression of hyperactivated motility depends upon the presence of calcium in the external media (Yanagimachi and Usui, 1974; Fraser, 1977; Shams-Borhan and Harrison, 1981; Yanagimachi, 1982; Cooper, 1984). Furthermore, these observations have been extended by demonstrating that the elevation of cAMP levels which occurs during capacitation also depends upon the presence of extracellular calcium.

Incubation of caudal spermatozoa in the absence of calcium, but in the presence of the phosphodiesterase inhibitor IBMX, caused a significant ($P<0.01$) increase in the cAMP content of these cells, although the level attained did not reach that expressed by the same cell type in medium MT-1. However, this elevation of cAMP levels was not associated with an increase

in the proportion of cells exhibiting hyperactivated motility. Furthermore, the addition of dibutryl cAMP to spermatozoa in medium NAC also failed to cause the resumption of hyperactivation, despite presumably raising cAMP levels. These results may indicate that calcium has multiple actions in the induction of hyperactivation, stimulating the increase in cAMP, but also fulfilling a more fundamental role, possibly at the level of the axoneme (see below).

In keeping with the hypothesis that a causal relationship exists between cAMP levels and hyperactivation, is the observation that treatment of caudal spermatozoa in complete medium MT-1 with the phosphodiesterase inhibitor IBMX accelerates the onset of hyperactivated motility, as has been reported previously (Fraser, 1979; Mrsny and Meizel, 1980). Furthermore, this phenomena was associated with a substantially augmented increase in cAMP levels. Similar treatment with the membrane permeant analogue of cAMP, dbcAMP, produced effects analagous to, although less pronounced than those achieved with IBMX.

The calcium requirement for the onset of hyperactivation does not appear, however, to be the only action of this cation in the expression of sperm movement. Caudal spermatozoa challenged with the calmodulin antagonist calmidazolium suffer complete disruption of flagellar beat patterns, and never express hyperactivated motility. These findings are in agreement with those of Tash and Means (1982), who observed similar disruption of dog sperm motility following treatment with the anti-calmodulin drug W13, but contrast with the observations of both Peterson et al (1983), who showed only a slight decrease in the motility of boar spermatozoa following treatment with 4μM calmidazolium, and Serres and Kann (1984), who showed that calmodulin antagonists induced mature motility patterns in hamster caput spermatozoa. Peterson et al (1983) further reported that this drug stimulated calcium uptake. However, this study has shown that the influence of calmidazolium upon hamster sperm motility is not associated with any changes of intracellular calcium concentration as determined using the Quin-2 technique. The disparity between these observed effects of

calmodulin antagonists upon sperm motility may either reflect species differences or effects attributable to actions via mechanisms which do not involve calmodulin. The influence of calmidazolium on caudal sperm motility does not appear to be due to non-specific toxicity, as treated cells continued to show twitching movements reminiscent of caput sperm over a 7h incubation period.

This total inhibition of coordinated flagellar movement resulting from calmidazolium treatment indicates a fundamental role for calmodulin in the expression of sperm movement. The localization of calmodulin throughout the sperm tail (Weinman et al, 1986; Feinberg et al, 1981, 1983; Gordon et al, 1983; Jones et al, 1980) further implicates this protein in the regulation of sperm motility. Such an action may involve the dynein ATPases located on the outer doublet microtubules of the axoneme, as these enzymes are known to be both essential for sperm motility and to be calcium-calmodulin dependant (Blum et al, 1980; Hisanaga and Pratt, 1984).

The rise in cAMP content seen under normal capacitation conditions at a sperm density of $1x10^6$/ml is slightly reduced following treatment with calmidazolium. This decrease may indicate that the adenylate cyclase enzyme in hamster spermatozoa is calmodulin dependant, as has been reported for spermatozoa of the guinea pig (Hyne and Garbers, 1979b). However, this slight reduction may relate only to the lower percentage motility seen under these conditions. Regardless of the mechanisms involved, this reduction of cAMP content does not appear to be the primary means by which calmidazolium affects sperm motility, as the addition of the phosphodiesterase inhibitor IBMX to these incubations elevated cAMP content to levels significantly higher than those seen in MT-1 alone, whilst failing to elicit either the resumption of normal patterns of motility or the appearance of hyperactivation.

In conclusion, these experiments have shown that in the hamster, the calcium and calmodulin dependant expression of hyperactivated motility is preceded by a calcium dependant elevation of cAMP levels. Furthermore, the expression of coordinated sperm motility in this species appears to be

dependant upon the mediation of calcium/calmodulin in a manner which is independant of cAMP.

# Chapter 4. Mechanisms involved in the epididymal development of the ability to express an increase in cAMP levels.

## A. Introduction.

The experiments detailed in chapters 2 and 3 have shown that capacitation of spermatozoa from the cauda epididymis of the hamster is associated with a progressive, calcium-dependant increase in cAMP content which precedes the onset of hyperactivated motility. This rise in cAMP levels is not, however, expressed by caput spermatozoa incubated under identical conditions to capacitating caudal spermatozoa, despite the presence of calcium in the external medium. In contrast to the rise expressed by caudal spermatozoa, the cAMP content of caput spermatozoa decreases upon dilution and remains low throughout a seven hour incubation period.

The increases in calcium content during capacitation which have been implied from observed influxes of this cation (Singh et al, 1978; Triana et al, 1980; Singh et al, 1980) could account for the elevation of cAMP levels during capacitation, via an action upon the calcium-dependant adenylate cyclase (Kopf and Vacquier, 1985). Furthermore, as the cAMP levels expressed by caudal spermatozoa in NAC closely resemble the cAMP content of caput spermatozoa in MT-1, the inability of immature cells to express a rise in cAMP content may be due to insufficient calcium being transported into the cells interior.

However, it has been reported that the capacity for calcium uptake is in fact greater in immature spermatozoa from the caput epididymis (Hoskins et al, 1983). It may then be that the calcium sensitivity of the adenylate cyclase enzyme changes during epididymal transit. Determination of the responsiveness of sperm adenylate cyclase to stimulation is complicated by the fact that in this cell type, this enzyme does not respond to the usual stimulators of adenylate cyclase activity such as, fluoride, guanyl nucleotides, cholera toxin or forskolin (Braun and Dods, 1975; Herman et al, 1976; Chen and Boettcher, 1979; Garbers and Kopf, 1980; Stengel et al, 1982; Forte et al, 1983), as in spermatozoa, the adenylate cyclase system diifers from that of somatic cells in that it appears to lack a regulatory subunit (Stengel and Hanoune, 1984; Hildebrandt et al, 1985). However, it has

recently been demonstrated that spermatozoal form of the adenylate cyclase complex will respond to stimulation by artificial analogues of adenosine, such as 2-chloro- and 2-deoxy- adenosine (Aitken et al, 1986; Vijayaraghavan and Hoskins, 1986). Thus, the responsiveness of the adenylate cyclase in caput spermatozoa can be probed using these reagents.

The ATP content of spermatozoa from the rat and bull spermatozoa has been reported to increase during epididymal transit (Chulavatnatol et al, 1977; Hoskins et al, 1975). Therefore, low substrate concentration in caput spermatozoa could account for the inability of these cells to express the rise in cAMP levels shown by capacitating caudal spermatozoa.

Another factor which may be involved in changes in the synthesis of cAMP during epididymal transit is intracellular pH. Epididymal maturation in the bull has been reported to be associated with an increase in intracellular pH (Vijayaraghavan et al, 1985). As the adenylate cyclase enzyme in spermatozoa has been reported to be stimulated by increases in intracellular pH (Peterson et al, 1980), such a mechanism could account for the low cAMP levels expressed by caput spermatozoa. Furthermore, motility itself is enhanced by alkalinization of the sperm interior (Hansbrough and Garbers, 1981; Christen et al, 1982; Babcock et al, 1983), presumably through the activation of dynein ATPase. Thus an increase in internal pH during capacitation could provide the primary signal for the onset and development of hyperactivated motility, through effects upon both dynein ATPase and adenylate cyclase.

Therefore, to determine the mechanisms involved in the generation of the increase in cAMP content during capacitation, and the reasons why spermatozoa from the caput epididymis are unable to exhibit a rise in cAMP levels, four factors have been studied; intacellular calcium content, ATP - the substrate for adenylate cyclase, intracellular pH, and the ability of the adenylate cyclase enzyme to respond to stimulation.

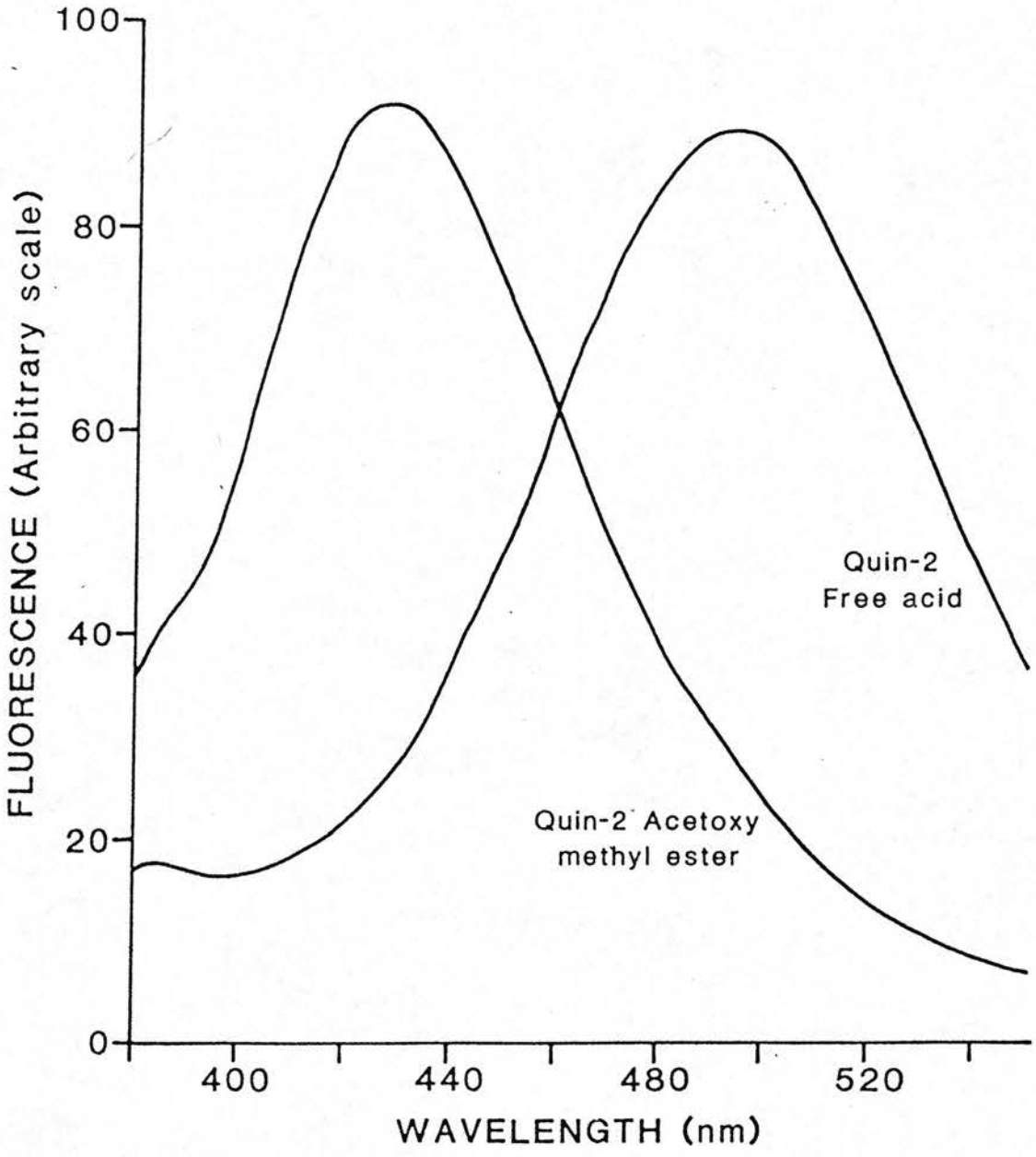



Figure 31. Emission spectra (scanned from 380 to 550nm) of Quin-2 tetracetoxymethylester (fluoresence peak 429nm), and Quin-2 free acid (fluoresence peak 492nm). Fluorescence intensity is plotted on an arbitrary scale.

## B. Materials and Methods.

Both the media employed in this study and the techniques utilised for; isolation of spermatozoa, extraction of cAMP, determination of cAMP levels and statistics are as outlined in chapter 2. Motility assessment was performed as outlined in chapter 3.

i. Intracellular Calcium Measurements. The intracellular concentration of free extramitochondrial calcium was assessed using the fluorescent calcium indicator Quin-2 (Tsien et al, 1982), using a modification of the methodology outlined by Irvine and Aitken (1986).

Spermatozoa were released into medium MT-1, and adjusted to a cell density of $4x10^6$/ml. Twenty minutes after initial dilution, Quin-2-tetracetoxymethylester [in dry dimethyl sulfoxide (DMSO)] was added at a concentration of 50μM, whilst DMSO alone was added to controls. The sperm suspensions were then incubated at 37°C (in 5% $CO_2$ in air) for 20 minutes, diluted 1:3 with fresh media, and subsequently incubated for a further 60 minutes.

This incubation time was chosen as a result of monitoring the loading and de-esterification of Quin-2 in caudal spermatozoa.This can be easily quantified as the membrane permeant tetracetoxymethylester has a characteristic emission spectrum with a fluorescence maximum at 429nm, whilst the free acid (which is membrane impermeant, and is produced intracellularly via hydrolysis by endogenous esterases) fluoresces maximally at 492nm (Fig 31). Sperm cells were loaded with Quin-2 and incubated in MT-1. At various time points, aliquots of the sperm suspension were removed and the cells washed twice by centrifugation at 250g for 5mins, before being lysed with Triton X-100 to release the intracellular Quin-2 into solution. The lysed cells were then removed from the solution by centrifugation, and the supernatant, containing the Quin-2, was used to assess the hydrolyzed state of the intracellular Quin by measurement of its

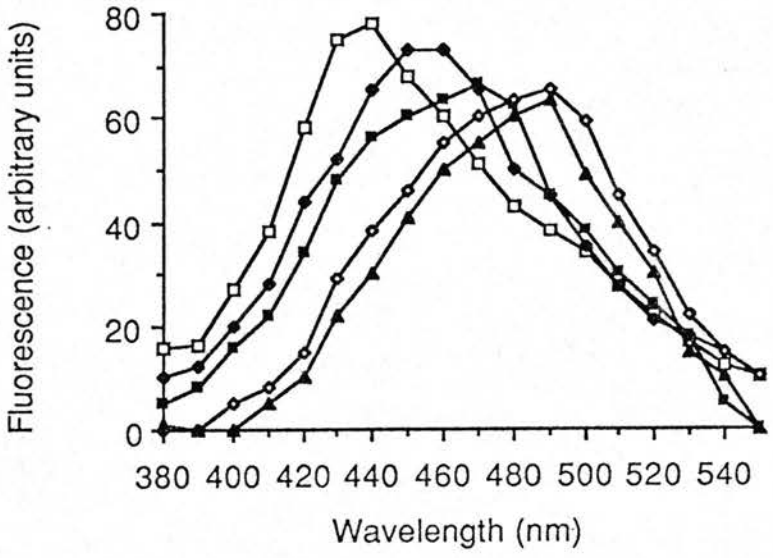



Figure 32. Emission spectra (scanned from 380 to 550nm) of the 3000g supernatant from triton x-100 lysed spermatozoa from the cauda epididymis of the hamster, incubated in the presence of Quin-2 tetracetoxymethylester for 20 (open squares), 40 (closed diamonds), 60 (closed squares), 80 (open diamonds), or 100 (closed triangles) mins. Fluoresence intensity is plotted on an arbitrary scale.

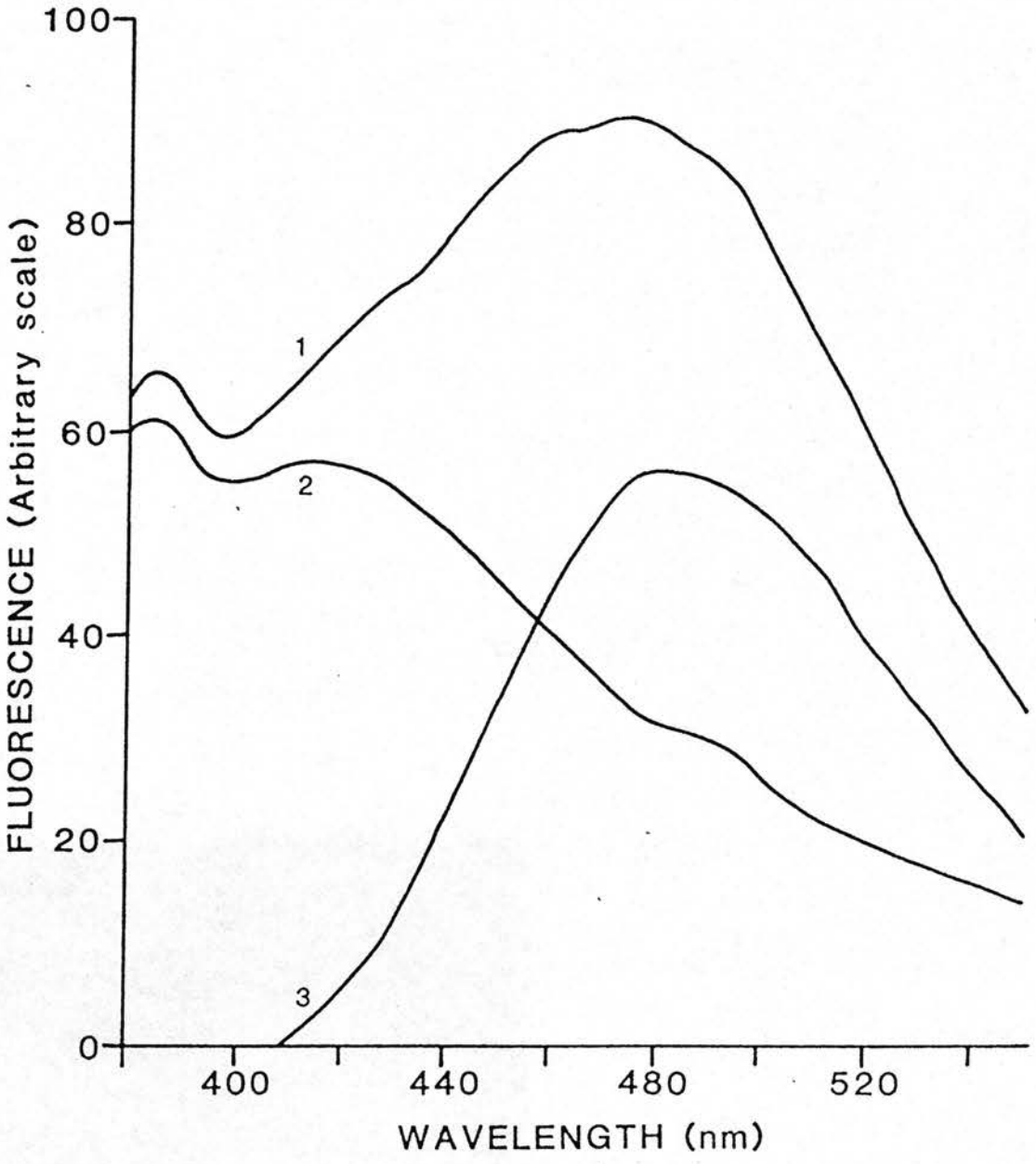



Figure 33. Emission spectra (scanned from 380 to 550nm) of spermatozoa from the cauda epididymis of the hamster incubated in the presence of Quin-2 tetracetoxymethylester (1) or DMSO (2) for a total of 80 mins. Spectrum 3 has been produced by subtracting the control spectrum (2) from the Quin spectrum (1). Fluoresence intensity is plotted on an arbitrary scale.

emission spectrum. From from such determinations it was apparent that complete hydrolysis has occured after a total incubation period of 80mins (Fig 32).

For determination of free intracellular calcium content, spermatozoa loaded with Quin according to the protocol outlined above were washed twice by centrifugation at 250g for five mins to remove extracellular Quin and finally resuspended in fresh medium at a concentration of $1.0x10^6$/ml. Fluorescence measurements were performed in either a Baird or a Perkin-Elmer spectrofluorimeter, with the excitation wavelength set at 339nm and the emission wavelength at 492nm. The sperm suspension was maintained at 37°C in a quartz cuvette whilst the determinations were performed. The entry of Quin-2 into the cells was checked by observing the shift in the fluorescence maximum from 430 to 492nm. Intracellular calcium content was determined by the addition of 0.5% v/v Triton X-100, followed by 10mM EGTA (giving $F_{max}$ and $F_{min}$ respectively).

In contrast to the situation in the human (Irvine and Aitken, 1986), hamster spermatozoa were found to emit significant levels of autofluorescence. To compensate for this effect, the F, $F_{max}$ and $F_{min}$ values measured in Quin loaded spermatozoa were adjusted by subtracting the fluorescence signal produced by identically treated cells incubated in D.M.S.O. alone (Fig 33). Calculation of intracellular calcium levels was achieved using the equation: $[Ca^{2+}]_i = K_d(F-F_{min})/(F_{max}-F)$, where $K_d$ = 115nM (Tsein et al, 1982).

ii. ATP Measurement. Extraction of ATP was achieved by mixing 25µl of sperm suspension with an equal volume of 0.01% trichloroacetic acid (TCA), followed by the addition of 850µl Tris-EDTA buffer (0.1M-Tris, 2mM-EDTA, pH 7.75). Bioluminescence was measured with a Berthold luminometer (Laboratory Impex Ltd, Twickenham, UK) after addition of 100µl luciferin-luciferase reagent (LKB-Wallace, Turku, Finland). Light emission was calibrated using standard solutions of ATP (LKB-Wallace).

iii. Intracellular pH Measurements. Intracellular pH was determined using the fluorescent pH indicator 2,7-Biscarboxyethyl-5(6)-carboxyfluorescein (BCECF - Research Development Corporation, Toronto, Canada). Twenty minutes after initial dilution, BCECF-tetraacetoxymethyl ester was added (5μg/ml in DMSO) to spermatozoa at a density of $4x10^6$/ml; DMSO alone was added to control incubations. Cell suspensions were then incubated at 37°C (in 5% $CO_2$/95% air) for 30 minutes to facilitate loading and de-esterification. Loaded spermatozoa were then washed twice by centrifugation at 250g for 5 mins to remove extracellular BCECF and finally resuspended in fresh media at a concentration of $1.0x10^6$/ml. Measurements were performed in a Perkin-Elmer spectrofluorimeter, with the excitation wavelength set at 500nM and the emission wavelength at 530nM. The suspension of spermatozoa was maintained at 37°C in a quartz cuvette whilst the determinations were performed. Fluorescence was calibrated by releasing the dye into the medium using 0.5% v/v Triton X-100, then titrating using 3.2% HCl (Rink et al, 1982; Grinstein et al 1984). Control incubations showed negligible levels of autofluorescence. Red shift effects were minimal over the pH range measured in this study.

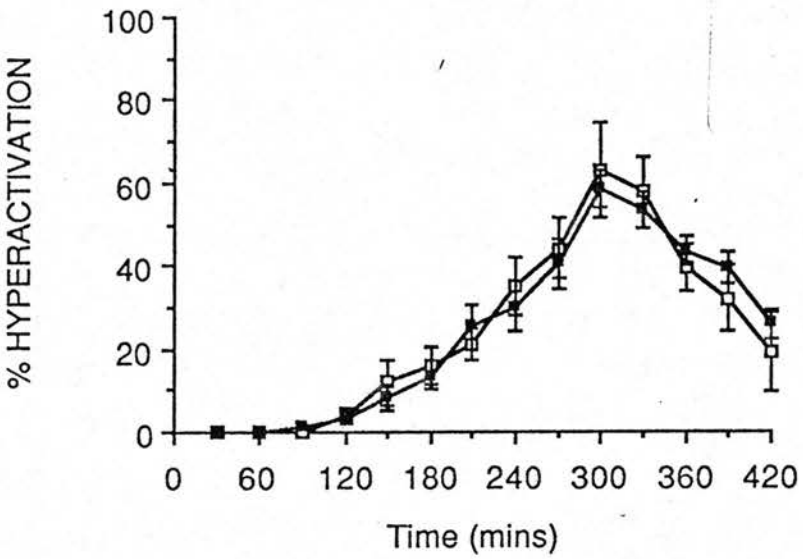



Figure 34. Percentage hyperactivated motility displayed by spermatozoa from the cauda epididymis of the hamster incubated in either MT-1 alone (closed squares), or in MT-1 plus 50μM Quin-2 (open squares) - see text for exact incubation details. Values shown represent the mean ± sem of six determinations.

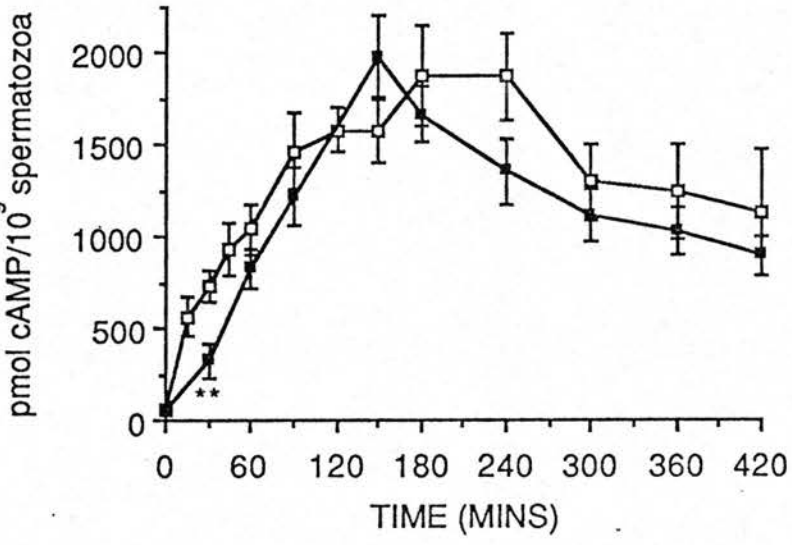



Figure 35. Cyclic AMP content (picomoles cAMP per $10^9$ spermatozoa), expressed by spermatozoa from the hamster cauda epididymis incubated over a 7h period in either medium MT-1 (open squares) or medium MT-1 plus 50μM Quin-2 (closed squares) - see text for incubation details. Significance levels are: **, $P<0.01$. Values shown are mean ± standard error, n=6.

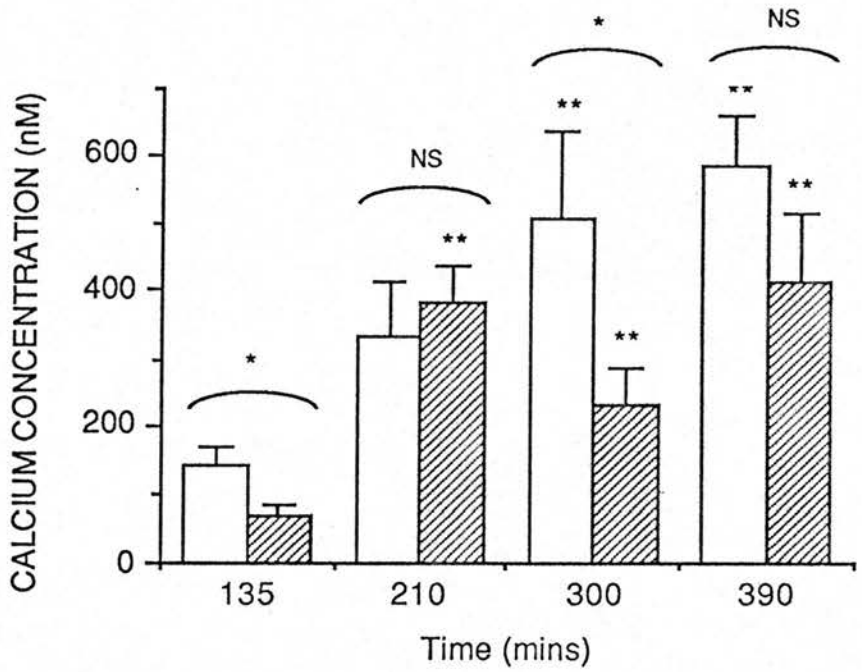



Figure 36. Free intracellular calcium content (nanomolar), determined at various times throughout a 7h incubation period, of hamster spermatozoa from the caput (open bars), or cauda (closed bars) epididymis incubated in medium MT-1 - see text for incubation details. Significance levels are: *, $P<0.05$; **, $P<0.01$. Values shown are mean ± standard error, n=6.

## C. Results.

### i. Quin-2.

a. Effect upon hyperactivation. As Quin-2 is a calcium chelating agent, and as the expression of hyperactivated motility is known to be affected by alterations of intracellular calcium concentration, it was first determined whether cells loaded with Quin-2 would display similar levels of hyperactivated motility to spermatozoa incubated in MT-1 alone. Analysis of this pattern of motility revealed that spermatozoa incubated in the presence of Quin-2 expressed levels of hyperactivated motility which did not differ significantly those displayed in medium MT-1 (Fig 34).

b. Effect upon cAMP levels. As the rise in cAMP levels expressed by caudal spermatozoa has also been found to be affected by calcium concentration, It was also determined whether spermatozoa loaded with Quin-2 would exhibit similar rises in cAMP content to those cells incubated in MT-1 alone. It was found that cAMP levels after 30mins of incubation were significantly ($P<0.01$) lower in Quin loaded cells than in spermatozoa incubated in medium MT-1 (Fig 35). This may be due to a lowering of intracellular calcium levels. However, after this initial suppression, spermatozoa incubated in the presence of Quin-2 proceded to exhibit a progressive increase in cAMP content which did not differ significantly that expressed by spermatozoa in medium MT-1.

c. Intracellular Calcium Determinations. Following the loading protocol outlined above, the initial determination of cytosolic calcium concentration was made after 135mins of incubation (Fig 36). At this time, the internal calcium content of caudal spermatozoa was 66.8 ± 17.0nM, significantly ($P<0.05$) lower that of caput spermatozoa (143.8 ± 26.6nM). During the subsequent incubation of both cell types there was a significant ($P<0.01$) increase in intracellular calcium levels. In the case of caput

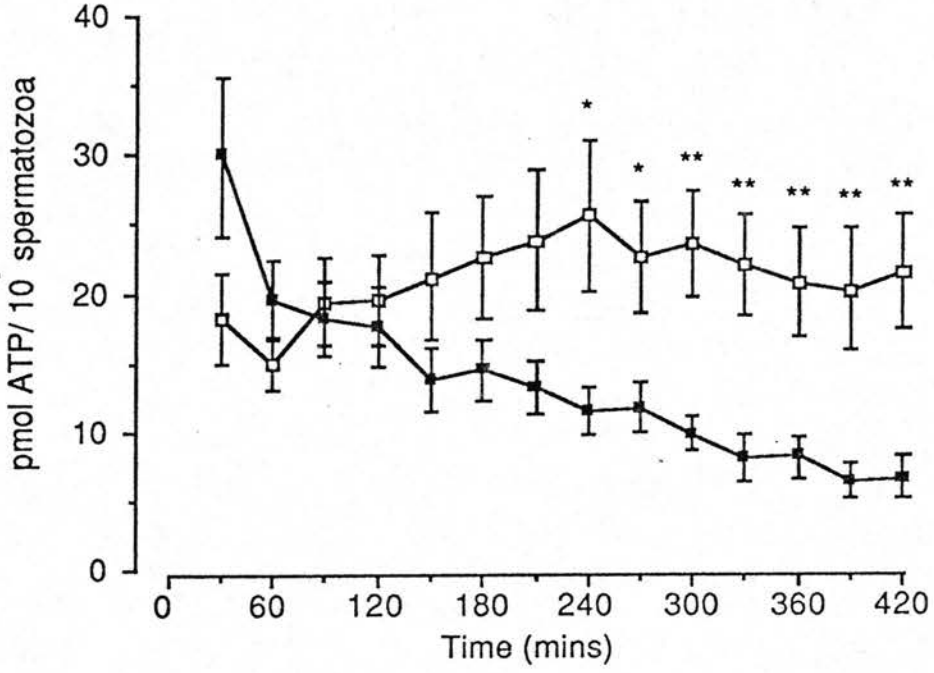



Figure 37. ATP content (picomoles per $10^6$ spermatozoa) of hamster spermatozoa from the caput (open squares), or cauda (closed squares) epididymis. Incubations were performed in medium MT-1 over a 7h time period, at a sperm density of $1x10^6$/ml. Significance levels are: *, $P<0.05$; **, $P<0.01$. Values shown are mean ± standard error, n=6.

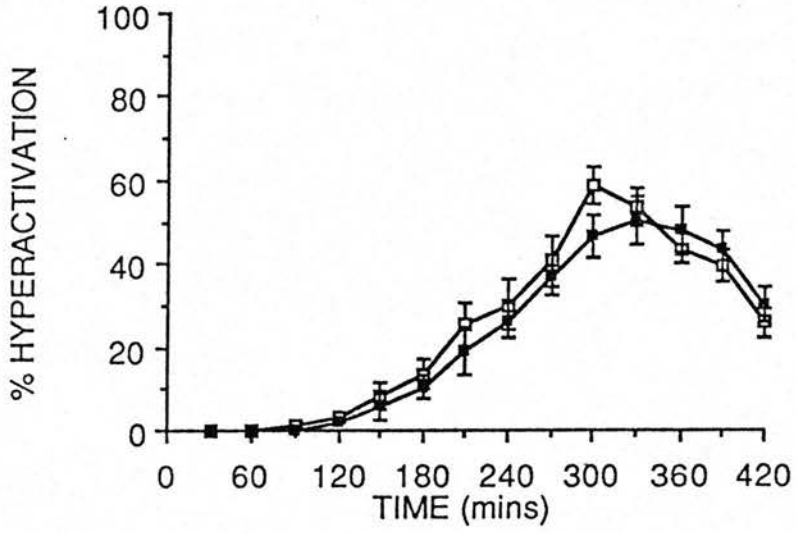



Figure 38. Percentage hyperactivated motility displayed by spermatozoa from the cauda epididymis of the hamster incubated in either MT-1 alone (closed squares), or in MT-1 plus 5µg/ml BCECF (open squares) - see text for exact incubation details. Values shown represent the mean ± sem of six determinations.

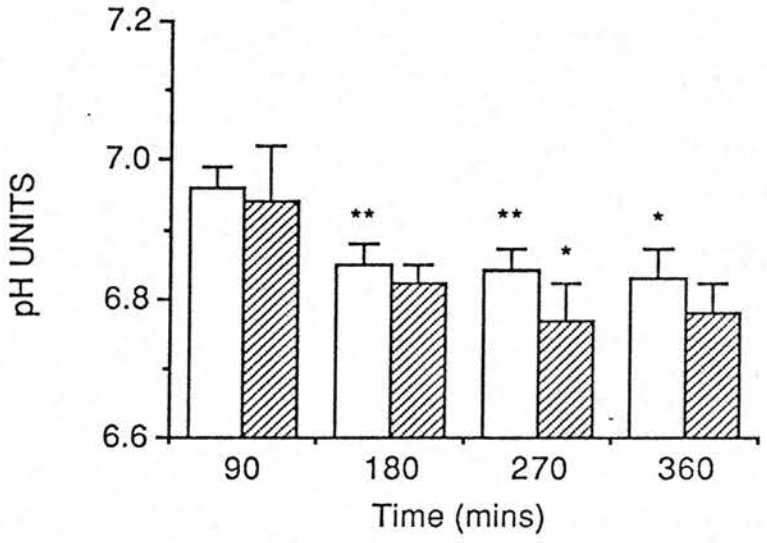



Figure 39. Intracellular pH, determined at various times throughout a 7h incubation period, of hamster spermatozoa from the caput (open bars), or cauda (closed bars) epididymis, incubated in medium MT-1. Significance levels are: *, $P<0.05$; **, $P<0.01$. Values shown are mean ± standard error, n=6.

epididymal spermatozoa, this increase was progressive in nature, reaching a peak after 390mins of incubation. Caudal epididymal spermatozoa showed a transient fall in intracellular calcium levels between 210 and 300 mins of incubation, when peak levels of hyperactivation are being expressed, such that at this time point the calcium levels in caput and caudal spermatozoa differed significantly ($P<0.05$).

ii. ATP Content. After 30mins of incubation, ATP levels in caudal spermatozoa are slightly, but insignificantly higher than in caput cells ($30.0 \pm 5.7$ vs $18.4 \pm 3.3$). As the incubation proceded, the ATP content of caudal spermatozoa progressively declined, whilst caput levels remained unchanged (Fig 37). Thus, after 240mins of incubation, caudal ATP content was significantly ($P<0.05$) lower than in caput spermatozoa ($25.6 \pm 5.4$ vs $11.6 \pm 1.7$), and by 7h after dilution, caudal levels had dropped still further to $7.0 \pm 1.5$ ($P<0.01$).

**iii. BCECF.**

a. Effects upon hyperactivation. The effects of this reagent upon various cell types have been quantified to a much greater extent than for Quin-2. It has been shown that loading cells with BCECF does not alter significantly their internal pH (Rink et al, 1982; Grinstein et al, 1984). However, the percentage hyperactivation displayed by caudal spermatozoa incubated in medium MT-1 containing BCECF was determined. When compared to the levels of this motility pattern exhibited in MT-1 alone, no significant difference was found (Fig 38).

b. Intracellular pH. Following the loading protocol outlined above, the first determination of internal pH was made after 90 mins of incubation. At this time, an internal pH of $6.937 \pm 0.064$ was measured in caudal spermatozoa. Continuation of these measurements over the period of hyperactivation revealed that a statistically significant ($P<0.05$) decrease in

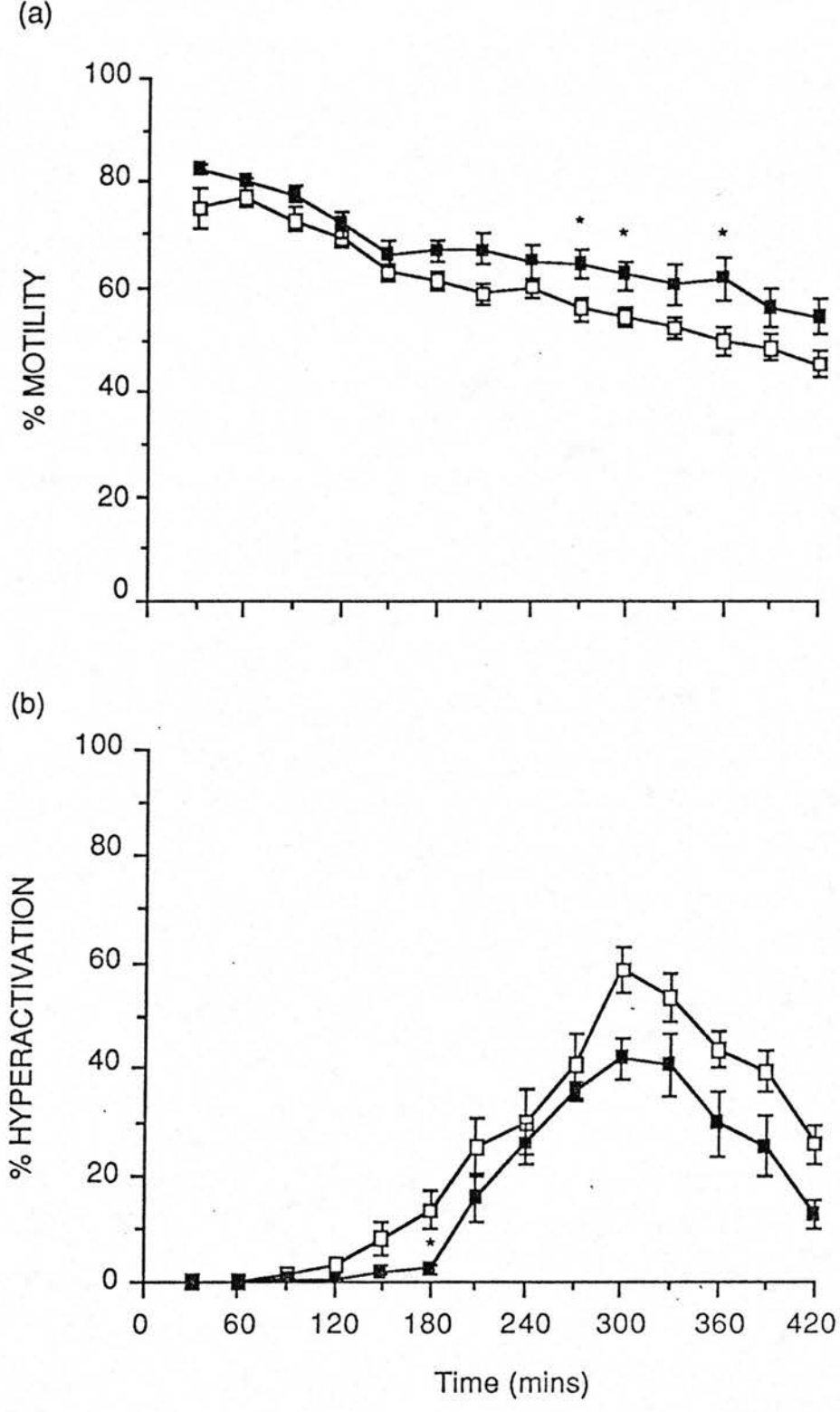



Figure 40. Percentage motility (a) and percentage hyperactivated motility (b) expressed by spermatozoa from the hamster cauda epididymis incubated at a density of $1x10^6$/ml for a 7h period in either medium MT-1 (open squares), or medium MT-1 plus 75μM 2-deoxyadenosine (closed squares). Significance levels are: **, $P<0.01$. Values shown are mean ± standard error, n=6.

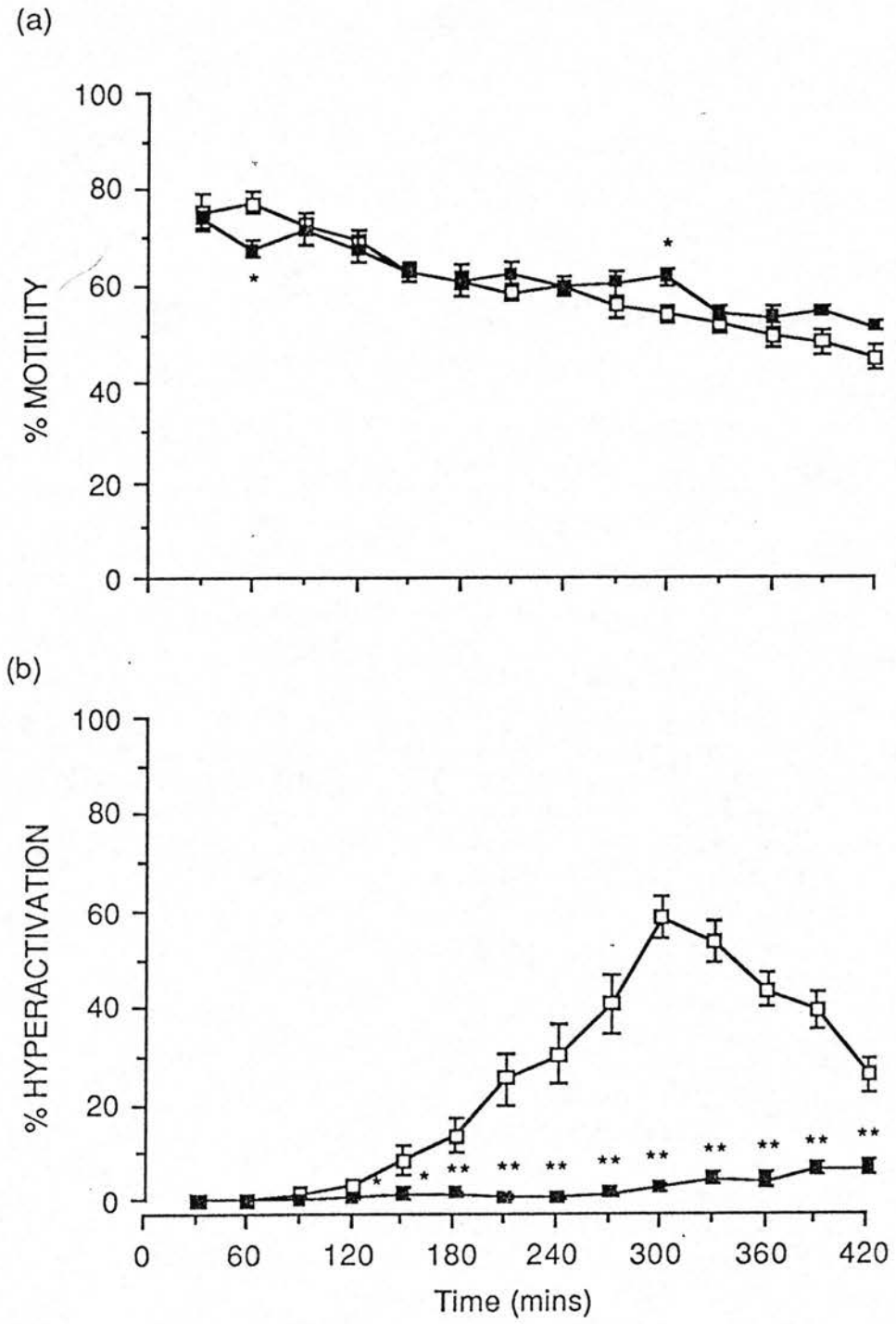



Figure 41. Percentage motility (a) and percentage hyperactivated motility (b) expressed by spermatozoa from the hamster cauda epididymis incubated at a density of $1x10^6$/ml for a 7h period in either medium MT-1 (open squares), or medium MT-1 plus 2.5mM 2-deoxyadenosine (closed squares). Significance levels are: *, $P<0.05$; **, $P<0.01$. Values shown are mean ± standard error, n=6.

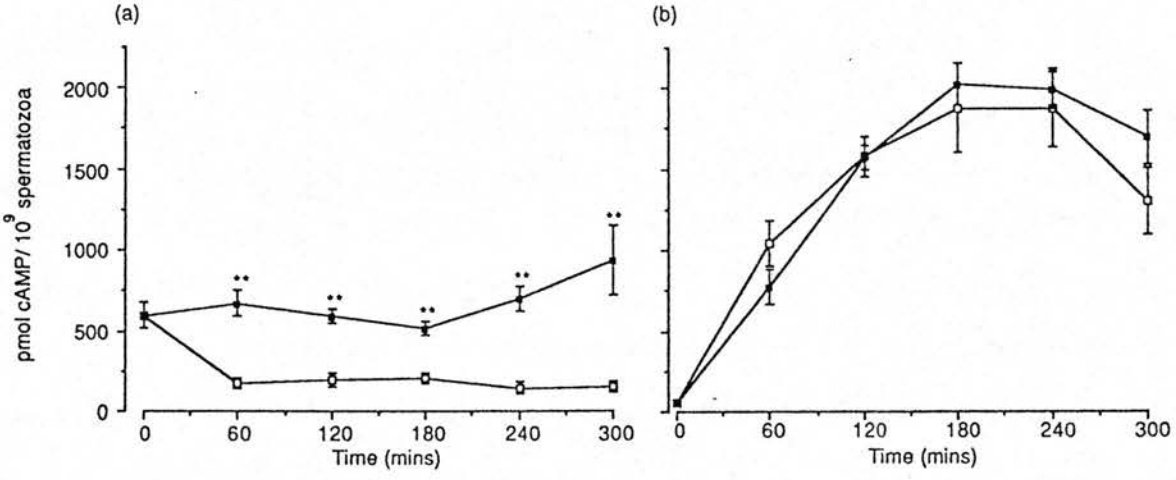



Figure 42. Cyclic AMP content (picomoles cAMP per $10^9$ spermatozoa) expressed by spermatozoa from the hamster caput (a), or cauda (b) epididymis, incubated at a density of $1x10^6$/ml over a 7h period in either medium MT-1 (open squares), or medium MT-1 plus 75µM 2-deoxyadenosine (closed squares). Significance levels are: **, $P<0.01$. Values shown are mean ± standard error, n=6.

[pH]i had occured after 4.5h of incubation, by which time values had dropped to 6.769 ± 0.045 (Fig 39). Parallel analysis of caput spermatozoa revealed that the intracellular pH of this cell type did not differ significantly from the values determined for caudal spermatozoa. These immature cells also expressed a significant ($P<0.01$) reduction in internal pH over time.

**iv. 2-deoxyadenosine.**

a. Motility. Caudal spermatozoa incubated in medium MT-1 in the presence of 75μM 2-deoxyadenosine (2-DA) showed a slight, but significant ($P<0.05$) increase in percentage motility when compared to similar spermatozoa incubated in MT-1 alone (Fig 40a). In contrast, this agent caused a significant ($P<0.05$) decrease in the percentage of live spermatozoa exhibiting hyperactivation (Fig 40b), despite having no effect on the rise in cAMP (see below).

At the higher concentration of 2.5mM, 2-DA had no consistent effect on percentage motility, levels being alternately significantly ($P<0.05$) lower after 1 h of incubation, then significantly ($P<0.05$) enhanced after 5 h of incubation (Fig 41a). However, at this dose, the percentage of spermatozoa expressing hyperactivated motility was severly attenuated, with levels being consistently significantly ($P<0.01$) lower than in MT-1 alone (Fig 41b).

b. Cyclic AMP. When added at a concentration of 75μM, 2-DA elevated the cAMP content of caput spermatozoa to levels significantly ($P<0.01$) higher than those normally associated with this cell type (Fig 42a). However, this reagent had no significant effect upon the characteristic increase in cAMP levels expressed by caudal spermatozoa during capacitation (Fig 42b).

When used at the higher concentration of 2.5mM, it was found that sufficient quantities of this reagent were carried over with the spermatozoa through the wash procedure to cross-react with the cAMP antisera. In order to determine the effects of this dose of 2-DA on the cAMP content

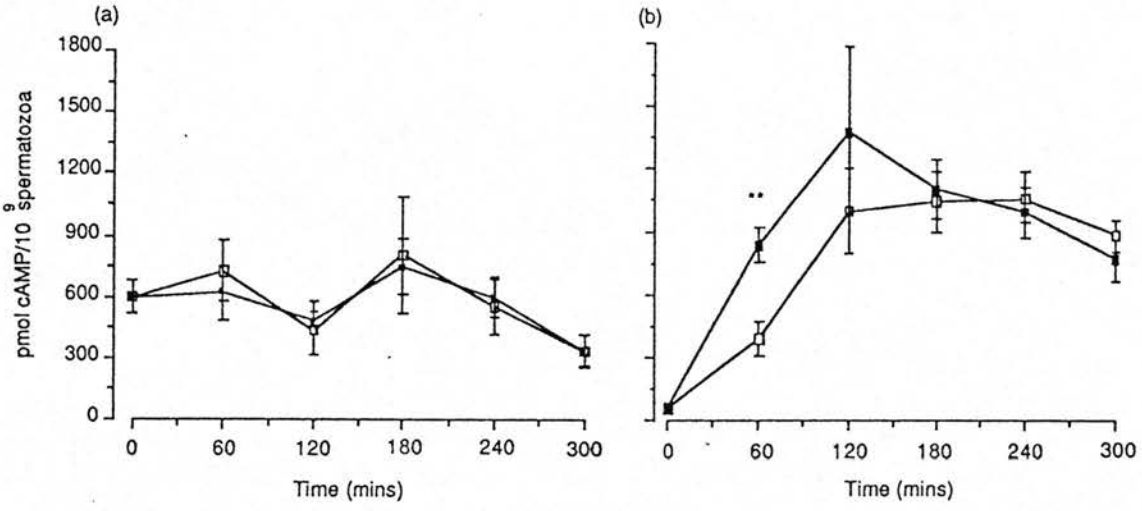



Figure 43. Cyclic AMP content (picomoles cAMP per $10^9$ spermatozoa) expressed by spermatozoa from the hamster caput (a), or cauda (b) epididymis, incubated at a density of $1x10^6$/ml over a 7h period in either medium MT-1 (open squares), or medium MT-1 plus 2.5mM 2-deoxyadenosine (closed squares). Significance levels are: **, $P<0.01$. Values shown are mean ± standard error, n=6.

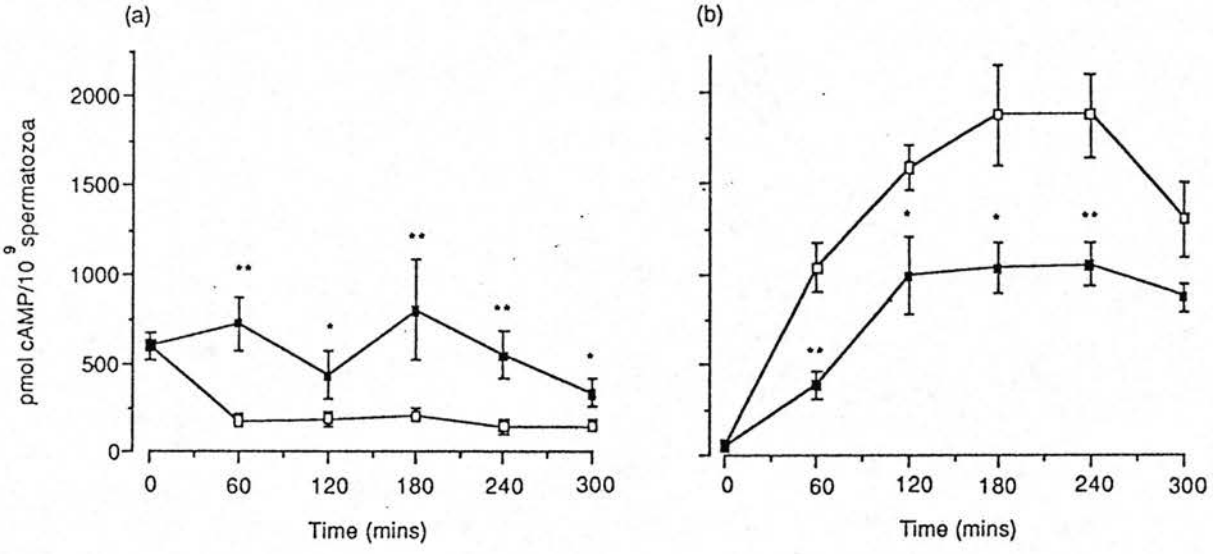



Figure 44. Cyclic AMP content (picomoles cAMP per $10^9$ spermatozoa) expressed by spermatozoa from the hamster caput (a), or cauda (b) epididymis, incubated at a density of $1x10^6$/ml over a 7h period in medium MT-1, undergoing either one (open squares), or two (closed squares) rounds of centrifugation at 250g for 5 mins. Significance levels are: *, P,0.05; **, P<0.01. Values shown are mean ± standard error, n=6.

of spermatozoa, an additional wash step was included. The amount of 2-DA remaining in the medium after two cycles of centrifugation did not cross-react with the cAMP antiserum. When compared to the cAMP content of control spermatozoa washed twice in MT-1, 2.5mM 2-DA was found to have no effect upon the cAMP content of caput spermatozoa (Fig 43a), but caused a significant ($P<0.01$) elevation of the cAMP levels in caudal spermatozoa after 60 mins of incubation (Fig 43b).

v. Centrifugation effects. In the course of the previous experiment, spermatozoa from both the caput and cauda epididymis were washed twice prior to extraction of cAMP. This procedure involved a repetition of the centrifugation step (250g, 5mins). Comparison of the cAMP content of spermatozoa treated in this manner to the levels expressed by cells washed with the normal single centrifugation revealed that the cAMP levels of caput spermatozoa were significantly ($P<0.01$) stimulated by additional centrifugation (44a), whilst this treatment caused a significant ($P<0.01$) decrease in the cAMP content of by caudal spermatozoa (44b).

## D. Discussion.

The progressive elevation of cAMP levels which is expressed by capacitating spermatozoa from the cauda epididymis of the hamster must reflect a change in the ability of these cells to generate this nucleotide. Capacitation in vitro is known to be accompanied by a net influx of calcium (Singh et al, 1978; Triana et al, 1980; Singh et al, 1980) and an increase in adenylate cyclase activity (Morton and Albagli, 1973; Stein and Fraser, 1984). Furthermore, increases in calcium content are known to activate sperm adenylate cyclase (Kopf and Vacquier, 1985). As caudal spermatozoa do not express this progressive rise in cAMP content when incubated in calcium-free media, it is possible that the stimulus for an increase in cAMP content may be an elevation of the levels of calcium available for the use of the adenylate cyclase complex. Similarly, the inability of caput spermatozoa to display a similar rise in cAMP levels, despite the presence of calcium in the external media, might have been due to a deficiency in their intracellular calcium concentration.

Using the fluorescent probe Quin-2, the intracellular calcium content of both capacitating caudal and immature caput spermatozoa has been directly quantified to determine whether; a) the calcium dependant rise in cAMP levels observed during capacitation is associated with an increase in free intracellular calcium content, and b) the low cAMP levels expressed by caput spermatozoa reflect an inability on the part of these immature cells to transport sufficient calcium across the plasma membrane.

Using this technique, it has been shown that the the rise in caudal cAMP content is temporally related to an elevation of the free intracellular calcium concentration in these cells. Furthemore, when cAMP levels reach a plateau after three hours of incubation, a similar constancy is seen in the calcium content of these cells. It can be concluded, therefore, that the rise in cAMP levels which occurs during capacitation results from a progressive stimulation of adenylate cyclase activity induced by increasing levels of intracellular calcium.

However, the free cytoplasmic calcium content of caput spermatozoa incubated under identical conditions and over the same time period, is either significantly ($P<0.05$) higher than, or not significantly different from the calcium levels in caudal spermatozoa. These observations are in agreement with the findings of Hoskins et al (1983), who showed that spermatozoa from the caput epididymis of the bull have a greater capacity for calcium uptake than caudal cells. Thus, as both cell types show an increase in intracellular calcium concentration over time, some factor other than calcium availability must prevent the elevation of cAMP levels in caput spermatozoa.

It is interesting to note that, when compared to the free calcium content of caput spermatozoa, the levels of this cation in caudal spermatozoa decrease significantly ($P<0.05$) after 5h of incubation, concomitant with the expression of peak levels of hyperactivation. This observation may indicate the activation of a cAMP-dependant calcium extrusion mechanism in these mature cells (Feinstein et al, 1983; Zavioco and Feinstein, 1984; Bradley and Forrester, 1985), compared to the apparently passive rise in the calcium content of caput spermatozoa.

Analysis of the literature reveals conflicting evidence concerning the effects of epididymal transit on the ATP content of spermatozoa. Concentrations of this nucleotide have been reported to increase (Chulavatnatol et al, 1977), or to remain constant (Ke and Tso, 1982) in rat spermatozoa; to rise in spermatozoa from the bull (Hoskins et al, 1975), or to fall in the guinea pig (Frenkel et al, 1973). However, none of these studies were performed longitudinally. To determine whether lower substrate concentration in caput spermatozoa could account for the inability of these cells to express the rise in cAMP levels shown by capacitating spermatozoa, the ATP content of both caput and caudal spermatozoa has been measured over a 7h incubation period. These measurements have revealed that although ATP levels are initially slightly, but insignificantly, higher in caudal spermatozoa, the ATP content of these cells declines as the incubation procedes, presumably reflecting the utilization of this nucleotide for the expression of flagellar movement. Similar decreases in intracellular ATP

content have previously been reported to occur in capacitating hamster spermatozoa (Rogers and Morton, 1973). Eventually, the ATP content of caudal spermatozoa fell to levels significantly ($P<0.01$) lower than those shown by caput spermatozoa, in concert with the significant increase in cAMP levels. Thus, lack of substrate availability does not account for the inability of caput spermatozoa to express the rise in cAMP levels associated with capacitation.

As both sperm adenylate cyclase activity (Peterson et al, 1980) and sperm motility (Hansbrough and Garbers, 1981; Christen et al, 1982; Babcock et al, 1983) are stimulated by increases in internal pH, a longitudinal study of the steady state pH of caudal spermatozoa was performed to investigate the possibility that the onset and development of hyperactivated motility during capacitation may reflect an increase in intracellular pH. Using the fluorescent probe BCECF, it has been shown that intracellular pH does not increase during capacitation, suggesting that the expression of hyperactivated motility is independant of this particular intracellular control mechanism.

Furthermore, the intracellular pH of caput spermatozoa incubated under identical conditions has been measured. In contrast to the situation in the bull (Vijayaraghavan et al, 1985), no significant difference in internal pH exists between these different cell types, implying that the inability of hamster caput spermatozoa to express an increase in cAMP levels is not due to differences in intracellular pH. This would also suggest that the initiation of motility upon dilution does not involve an elevation of internal pH as has been suggested for spermatozoa from the rat (Wong et al, 1981; Treetipasatit and Chulavatnatol, 1982; Wong and Lee, 1983), bull (Acott and Carr, 1984) and sea urchin (Schackman et al, 1981; Christen et al, 1982; Lee et al, 1983). The measured decrease of internal pH which occured during the prolonged incubation of both caput and caudal spermatozoa may be caused by acidification of the external media resulting from extensive lactate production, as has been reported for bull spermatozoa (Smith et al, 1985).

Although the adenylate cyclase of caput spermatozoa appears not to respond to the calcium present in the external media, this enzyme is not totally unresponsive to stimulation in this cell type. The adenosine analogue, 2-deoxyadenosine, one of the few reagents known to stimulate the spermatozoal form of adenylate cyclase, when used at a concentration known to increase the activity of this enzyme in spermatozoa from other species (Vijayaraghavan and Hoskins, 1986), induced a significant ($P<0.01$) increase in the cAMP content of caput spermatozoa. The inability of higher concentrations of 2-DA to stimulate cAMP levels in caput spermatozoa may be due desensitization of the adenylate cyclase complex (Aitken et al, 1986). The slight inhibition of hyperactivation which occurred at a concentration of 75µM may reflect an action upon the intracellular calcium concentration as has been detailed for other cell types (Henon and McAffee, 1983; Dunwiddie, 1984). Such effects may also be responsible for the severe inhibition of hyperactivated motility which resulted from treatment with 2.5mM 2-DA. However, this suppression of motility was not due to an inhibition of adenylate cyclase activity, as has been reported to occur when spermatozoa from the bull are treated with this drug (Brown and Casillas, 1984).

Synthesis of cAMP in caudal spermatozoa may be responsive to stimulation by some agent, such as adenosine (Stein et al, 1986) which is inactive with respect to caput cells. However, the only change in adenylate cyclase activity documented to accompany epididymal maturation is the apparent loss of sensitivity to forskolin exhibited by bovine spermatozoa (Vijayaraghavan and Hoskins, 1985). In view of the conflicting reports of the sensitivity of sperm adenylate cyclase activity to forskolin, the implications of this observation are unclear. The gradual rise in cAMP levels expressed by caudal spermatozoa does not, however, suggest an active stimulation. Such an effect would be expected to produce a rapid response. Instead, the dynamics of the rising nucleotide levels would appear to reflect a progressive increase in activity.

It is interesting to note that the inclusion of an extra centrifugation

step, a process known to destabilize membranes, caused a significant ($P<0.01$) stimulation of the cAMP content of caput spermatozoa, whilst significantly ($P<0.01$) decreasing the cAMP levels in caudal spermatozoa. The loss of cholesterol from the plasma membrane of spermatozoa during epididymal maturation (Scott et al, 1967; Lavon et al, 1970; Terner et al, 1975; Adams and Johnson, 1977) implies that the stability of these membranes is decreasing (Johnson, 1975). As adenylate cyclase activity is known to be intimately related to membrane fluidity and to be stimulated by membrane perturbing agents (Salesse and Garnier, 1984), the stimulatory effects of centrifugation upon the cAMP content of caput spermatozoa may indicate that the inability of these immature cells to express a rise in cAMP levels is due to an inhibition of adenylate cyclase activity as a result of the lack of protein mobility within the plasma membrane

In conclusion, these experiments have shown that both the increase in cAMP levels and the expression of hyperactivated motility by caudal spermatozoa from the hamster epididymis appears to be controlled by an elevation of cytoplasmic calcium concentration, which accompanies capacitation in this species. However, the inability of caput spermatozoa to express a similar rise in cAMP levels does not appear to be due to deficiences in their calcium or ATP content, or to a difference in internal pH.

# Chapter 5. General Discussion

A. Development of coordinated motility. Mammalian spermatozoa are essentially immotile when they leave the testis, and are capable of displaying only uncoordinated twitching movements upon dilution. The ability to initiate both coordinated and progressive movement is gradually attained as spermatozoa pass through the caput and corpus epididymis. Functionally mature spermatozoa are then stored in a quiescent state in the cauda epididymis, prior to ejaculation.

In the bull, acquisition by spermatozoa of the capacity for coordinated progressive movement is correlated with an increase in their cAMP content (Hoskins et al,1974). Furthemore, flagellar activity can be stimulated in caput spermatozoa from this species by incubation in the presence of phosphodiesterase inhibitors (Hoskins et al, 1975). Coupled with the large body of evidence which relates artificial elevations of cAMP levels to an enhancement of sperm motility (Drevius, 1971, 1972; Garbers et al, 1971a, b, 1973b; Hoskins, 1973; Bunge, 1973; Haesungcharern and Chulavatnatol, 1973; Frenkel et al, 1973b; Schoenfeld et al, 1973, 1975; Morton et al, 1974; Hommonnai et al, 1976; Wyker and Howards, 1977; De Turner et al, 1978; Paz et al, 1978; Kann and Serres, 1980), a direct causal link has been proposed to exist between the cAMP content of epididymal spermatozoa and their capacity for movement (Garbers and Kopf, 1980; Tash and Means, 1983). In fact, in the light of the more recent demonstration that the cAMP levels in ram spermatozoa also increase during epididymal transit (Amann et al, 1982), and that the generation of flagellar movement in both spermatozoa and a number of other motile systems requires the cAMP-dependant phosphorylation of an axonemal protein (Tash et al, 1984), this assumption has become a basic tenet of motility research.

However, in direct contradiction to the accepted model, it has been demonstrated that intact spermatozoa from the cauda epididymis of the hamster (this study) and rat (Del Rio and Raisman, 1978; this study) are capable of expressing coordinated and progressive motility eventhough their cAMP content is less than that of caput spermatozoa. It would thus seem that the basal levels of cAMP present in the mature spermatozoa from

these species are sufficient to fulfill the requirements of this nucleotide for the induction of coordinated flagellar movement. The block which prevents caput spermatozoa from expressing mature motility patterns must therefore exist downstream from the requirement for cAMP. If the inability of spermatozoa from the caput epididymis of the hamster to express mature motility patterns is not due to low levels of cAMP, what other factors change during epididymal transit to enable these cells to display coordinated movement?

Although the flagellar system of spermatozoa is thought to undergo some modification during epididymal maturation (Mohri and Yanagimachi, 1980; Yeung 1984, 1986; White and Voglmayr, 1986), spermatozoa from the caput epididymis, which are immotile when intact, will exhibit coordinated flagellar movement upon demembranation and reactivation in a suitable medium (Mohri and Yanagimachi, 1980; Treetipasatit and Chulavatnatol, 1982; Yeung 1984, 1986; White and Voglmayr, 1986). These observations imply that the motor apparatus of these spermatozoa is already functionally assembled following spermatogenesis, and that some other factor must therefore change during epididymal transit.

As the only known role for cAMP in eukaryotic cells is the stimulation of protein phosphorylation via activation of protein kinase A (Rosen et al, 1975; Flockhart and Corbin, 1982), changes in the activity of this enzyme could thus affect the acquisition of motility in the epididymis. Although protein kinase activity has been found to increase during epididymal transit in the bull (Hoskins et al, 1974), levels of this enzyme have been reported both to rise (Pariset et al, 1985) and fall (Wooten et al, 1987) in the ram and to remain unchanged in the rat (Atherton et al, 1985) during epididymal maturation. However, care has to be exercised in the interpretation of these findings, as although sufficient protein kinase activity may be demonstrated in broken cell preparations of caput spermatoza, this enzyme may be inactivated in vivo.

Analysis of the products of protein kinase activity have revealed two phosphorylated proteins of 20 and 16Kd which appear exclusively in

spermatozoa from the cauda epididymis of the ram (Wooten et al, 1987). This contrasts with the situation in the rat, where five minor phosphorylated proteins were associated only with the axonemes of caput spermatozoa (Chulavatnatol et al, 1982). These conflicting reports emphasise the difficulties encountered when data obtained from different mammals are compared, as the factors which control the capacity for sperm movement may vary between species. A system may operate in the ram whereby the acquisition of motility during epididymal transit is initiated by the activation (phosphorylation) of specific "motility" proteins, which occurs when the cAMP content of spermatozoa reaches a level adequate to activate protein kinase A. Conversely, the substrate for these kinases may be absent in caput spermatozoa. The existence of such a situation is unlikely, however, as this would imply that maturing spermatozoa are capable of active protein synthesis, an ability which, it is generally accepted, spermatozoa do not possess (Mann, 1983). By contrast, in the rat (and possibly the hamster), where cAMP levels and protein kinase activity decrease during epididymal maturation, the acquisition of motility may require the dephosphorylation of certain proteins in caudal cells which inhibit the motility of caput spermatozoa.

The roles of cAMP and calcium in the regulation of flagellar motion are known to be closely related (Garbers and Kopf, 1980). Tash and Means (1983) forwarded the hypothesis that cAMP and calcium exert opposing actions in the regulation of sperm motility, respectively phosphorylating and dephosphorylating specific axonemal proteins, such as dynein ATPase (Tash and Means, 1982), with calcium negating the stimulatory effects of cAMP. Although evidence does exist, from experiments utilizing both intact and demembranated spermatozoa from a variety of species, that elevation of cAMP levels results in a stimulation of motility (see above), the effects of calcium on sperm motility vary widely, depending upon both the species examined and the experimental approach adopted. Direct evidence for a negative effect of calcium upon sperm motility comes from experiments utilising both intact (Davis, 1978; Singh et al, 1980; Fraser, 1977) and

demembranated (Mohri and Yanagimachi, 1980; Mohri and Yano, 1980, 1982; Gibbons and Gibbons, 1980; Tash and Means, 1982) spermatozoa. However, results from both cell models relate high (and probably supra-physiological) concentrations of calcium to reduced sperm viability.

Indirect evidence for an inhibitory role for calcium in the regulation of sperm movement comes from experiments in which calcium was removed from the external medium. In intact spermatozoa, Hong et al (1984) showed that incubation of ejaculated human spermatozoa in media rendered calcium free by the addition of either EDTA or EGTA, elicited a stimulation of sperm motility. However, the baseline motility exhibited by these spermatozoa when incubated in media containing normal levels of calcium appears to be sub-standard. These low levels of motility may be due to the inhibitory action of abnormally high levels of calcium within this population of asthenozoospermic patients. The stimulation seen in response to incubation in calcium free media may, therefore, be due to the normalizing effects of the chelating agents on intracellular calcium levels, rather than by complete removal of calcium from the spermatozoa.

Although the movement characteristics of intact, ejaculated human spermatozoa were shown not to differ between incubations performed in medium containing either 1.7mM or 10μM calcium, Aitken et al (1986) showed that the stimulation of motility which resulted from the elevation of cAMP levels following treatment with caffeine or 2-deoxyadenosine was significantly diminished in the presence of normal (1.7mM) levels of calcium. However, removal of calcium from the external medium severly inhibited the motility of intact spermatozoa from the hamster (Morita and Chang, 1970; Yanagimachi, 1982), rat (Miyamoto and Ishibashi, 1975; Davis, 1978) and mouse (Miyamoto and Ishibashi, 1975; Heffner et al, 1980; Heffner and Storey, 1981). Paradoxically, the reactivation of motility in demembranated spermatozoa is unaffected by the absence of this cation (Morton, 1973; Treetipasatit and Chulavatnatol, 1982, Mohri and Yanagimachi, 1980).

However, recent findings may provide an explanation for these discrepancies. Flagellar dynein adenosine triphosphatase (ATPase)

activity, known both to be essential for sperm motility and to be calcium-calmodulin dependant (Blum et al, 1980; Hisanaga and Pratt, 1984), is stimulated by Triton X-100 (Hisanaga and Pratt, 1984). Therefore, the calcium dependancy of intact-sperm motility may be circumvented in demembranated sperm models by the action of the detergent used to permeabilise these cells. Thus, the only discernable actions of calcium on the motility of these spermatozoa are the inhibitory effects caused by high levels of this cation.

Results from this study indicate that the presence of 1.7mM calcium in the external media is required for both the maintenance of sustained motility and the appearance of hyperactivation. Part of this calcium requirement is to stimulate the increased synthesis of cAMP during capacitation. Generation of sufficient levels of this compound appears to be involved with the onset of hyperactivation (see below). This study has also demonstrated that the free internal calcium concentration of caput spermatozoa from this species is neither too high, so that it inhibits sperm motility, nor too low to support sperm movement. However, through the use of the calmodulin antagonist calmidazolium, evidence has also been provided which suggests that the expression of coordinated motility itself is dependant upon a calcium and calmodulin dependant regulatory event downstream from the cAMP requirement for motility.

Calmodulin has been detected throughout the sperm flagellum (Jones et al, 1980; Feinberg et al, 1981, 1983; Gordon et al, 1983; Weinman et al, 1986) and, in fact, comprises the major acidic glycoprotein of spermatozoa (Brooks and Seigel, 1973). The demonstration that calmodulin antagonists disrupt sperm movement (Levin et al, 1981b; Peterson et al, 1983), and induce patterns of motility in mature spermatozoa which resemble those displayed by spermatozoa from the caput epididymis of the dog (Tash and Means, 1982) and hamster (this study) may indicate a primary involvement for this calcium-binding protein in the epididymal acquisition of the capacity for sperm movement. However, the demonstration that the intracellular concentration of calmodulin in ram spermatozoa

decreases as they pass through the epididymis (Pariset et al, 1985), may indicate a different role for this compound in this species. The effect of calmodulin on sperm motility may operate via activation of $Mg^{2+}$ ATPases on the outer doublet microtubules (Blum et al, 1980; Hisanaga and Pratt, 1984). Unfortunately, no data is available on the calmodulin levels associated with epididymal spermatozoa of the hamster.

Evidence from a variety of different species has shown that substances secreted by the epididymal epithelium modify the plasma membranes of spermatozoa as they pass through this organ (Hunter, 1969; Barker and Amann, 1971; Killian and Amman, 1973; Lea et al, 1978; Faye et al, 1980; Moore, 1980, 1981b; Jones et al, 1981; Wong and Tsang, 1982; Brooks and Tiver, 1983). Although many of these membrane alterations occur over the acrosomal surface (Kohane et al, 1979, 1980; Yanagimachi, 1981; Brooks and Tiver, 1983), and are thought to be involved in the development of the ability to recognize, bind to and penetrate the ovum (Saling, 1982; Orgebin-Crist and Fournier-Delpech, 1982; Fournier-Delpech et al, 1984; Cuasnicu et al, 1984), alterations of the flagellar membrane have also been documented (Lea et al, 1978; Vernon et al, 1982; Brown et al, 1983; Zeheb and Orr, 1984; Ellis et al, 1985; Olson et al, 1987). These changes are presumed to play some role in the epididymal development of the capacity for sperm movement, but this remains to be demonstrated. Forward motility protein, which is known to stimulate forward progression in bovine spermatozoa (Acott and Hoskins, 1978; Hoskins et al, 1978), has not been shown to bind specifically to the flagellum (Brandt et al, 1978; Acott and Hoskins, 1981). However, a recent report has indicated that the forward motility protein actually consists of two separate peptides. Although one binds to the head, the other has been localised specifically on the principal piece of the hamster sperm flagellum (Raynaud and Kann, 1986). This report provides the first evidence for a direct link between events at the level of the plasma membrane, and alterations of flagellar function. However, a causal relationship between these phenomena remains to be demonstrated.

It is known that the induction of motility in bovine caput epididymal

spermatozoa which results from treatment with either stimulators of adenylate cyclase or phosphodiesterase inhibitors can be potentiated by synchronous elevation of intracellular pH (Vijayaraghavan and Hoskins, 1985; Vijayaraghavan et al, 1985; Vijayaraghavan and Hoskins, 1986). Furthermore, the internal pH of bovine spermatozoa has been shown to increase during epididymal maturation (Vijayaraghavan et al, 1985). These observations indicate that, in the bull at least, control of intracellular pH represents a pivotal mechanism which governs the development of motility during epididymal transit. Such a hypothesis has a sound theoretical basis, in that sperm dynein ATPase activity, which is known to be essential for axonemal motion, has an alkaline pH optimum (Gibbons and Gibbons, 1972). However, measurements of the intracellular pH of caput and caudal spermatozoa from the hamster (see chapter 4) revealed no significant difference between these two different cell types. Thus, regulation of internal pH would not appear to occupy the same central position in the epididymal development of motility in the hamster, as it does in the bull.

Treatment of spermatozoa from the bovine caput epididymis with high concentrations of phosphodiesterase inhibitors (Hoskins et al, 1975; Acott et al,1983), or lower doses of these reagents in the presence of weak bases (Vijayaraghavan and Hoskins, 1985; Vijayaraghavan et al, 1985; Vijayaraghavan and Hoskins, 1986) results in the induction of a degree of coordinated motility not present in control cells. This stimulation of flagellar movement results from an elevation of cAMP levels and an increase of internal pH, both of which have been shown to be lower in bovine caput spermatozoa (Hoskins et al, 1974; Vijayaraghavan et al, 1985). However, reports that similar treatment of caput spermatozoa from the hamster also results in the induction of coordinated motility (Kann and Serres, 1980; Cornwall et al, 1986) are difficult to interpret in light of the findings in this study which show that cAMP levels decrease during epididymal maturation in the hamster, and that no significant difference exists between the internal pH of spermatozoa from the proximal and distal portions of the epididymis. Possibly the phosphodiesterase inhibitors utilized in these studies are

operating via mechanisms other than the phosphodiesterase enzyme itself, as has been implicated in other experimental situations employing these compounds (Tamblyn and First, 1977). Such effects may be directed towards altering the calcium homeostasis of the spermatozoa (Kopf et al, 1984).

Alterations in the ATP content of spermatozoa during epididymal maturation have also been implicated as a mechanism controling the acquisition of the capacity for coordinated movement, either as a result of levels being so high in caput spermatozoa that they inhibit sperm movement (Hoskins, 1973; Brokaw, 1984; Yeung, 1986, 1987), or being too low to support the energy requirements imposed by the expression of sperm movement (Hoskins et al, 1975; Chulavatnatol et al, 1977). However, the measurements performed in this study demonstrated that caudal spermatozoa from the hamster initially contain greater amounts of ATP than caput spermatozoa, but subsequently express both coordinated and hyperactivated motility when their ATP levels are significantly ($P<0.01$) lower than those of immature cells. These results thus discount a direct effect of ATP on the acquisition of motility in this species.

In conclusion, these experiments have shown that the epididymal maturation of hamster spermatozoa is not accompanied by an increase in cAMP levels, and that the acquisition of the capacity for movement which accompanies this process does not appear to be due changes in ATP content, intracellular calcium levels, or internal pH.

B. Development of hyperactivated motility. The shortening of capacitation time (Rosado et al, 1974; Toyoda and Chang, 1974b; Fraser, 1981; Aitken et al, 1983) and the accelerated onset of hyperactivation (Fraser, 1979; 1981; Mrsny and Meizel, 1980; Boatman and Bavister, 1984; Burkman, 1984; this study) which results from treatments which artificially elevate the cyclic AMP content of spermatozoa imply that in addition to its obligatory requirement for the induction of coordinated movement (Tash et

al, 1984), this nucleotide may play a more subtle, modulatory role in the expression of sperm motility (Tash and Means, 1982). The observed increase in adenylate cyclase (Morton and Albagli, 1973; Stein and Fraser, 1984), and the decrease in phosphodiesterase activity (Monks et al, 1986; Monks and Fraser, 1987) during capacitation further implicate a role for this nucleotide in the post ejaculatory modifications of sperm movement.

Although previous studies which measured the cAMP content of spermatozoa during capacitation failed to link changes in cAMP levels to the onset and expression of hyperactivated motility (Hyne and Garbers, 1979a; Stein and Fraser, 1984), possibly as a result of the masking effect of phosphodiesterase inhibitors present in epididymal plasma (this study), the experiments detailed in this thesis have shown that caudal spermatozoa from the hamster exhibit a rise in cAMP levels prior to displaying the characteristic flagellar movements of hyperactivation. Two lines of evidence for a causative association between cAMP and hyperactivation are:- 1) acceleration of the onset of hyperactivation following treatment of capacitating spermatozoa with phosphodiesterase inhibitors is associated with an enhancement of the increase in cAMP content; and 2) the removal of exogenously added calcium almost completely abolishes hyperactivation and concomitantly reduces cAMP levels. These observations suggest that cAMP is involved in the post-ejaculatory modification of sperm motility, although they do not provide conclusive proof for this hypothesis. However, assuming this theory is correct, by what means does cAMP manifest its actions?

It has been suggested that capacitation involves the synthesis of a critical level of cAMP (Fraser, 1981). As the only known action of this nucleotide in eukaryotic cells is the stimulation of protein phosphorylation via activation of protein kinase A (Rosen et al, 1975; Flockhart and Corbin, 1982), a certain concentration of cAMP may be required to trigger the phosphorylation of key proteins present in either the plasma membrane or the axoneme (Amann et al, 1982). However, although treatment with a phosphodiesterase inhibitor advanced the onset of hyperactivation,

spermatozoa incubated in the presence of such an agent did not express significant levels of hyperactivated motility when their cAMP content equalled that at which spermatozoa in MT-1 exhibited this movement pattern. This implies that the action of cAMP in the induction of hyperactivation may involve mechanisms other than those which occur in the expression of coordinated movement, in which this nucleotide is thought to operate directly upon the axoneme (Murofushi et al, 1986; Tash et al, 1986).

Increased levels of cAMP are known to antagonise calcium mobilisation (Berridge, 1984) and to maintain low levels of this cation in the cytoplasm of platelets, possibly through an action on ATP-dependant ion pumps (Feinstein et al, 1983; Zavoico and Feinstein, 1984; Bradley and Forrester, 1985). In view of the inhibitory influence of high levels of calcium upon sperm motility (see above; Breibart et al, 1985), and the fall in calcium content which occurs at the peak of hyperactivation, after cAMP levels have reached their zenith (this study), the possibility exists that cAMP may exert control over the cytoplasmic calcium content of these spermatozoa. Such an action could be mediated by effects upon $Ca^{2+}/Mg^{2+}$ ATPases located in the sperm plasma membrane (Feinstein et al, 1983; Zavoico and Feinstein, 1984; Bradley and Forrester, 1985), causing a decrease in the calcium concentration of the cell. Whether the action of cAMP in the control of hyperactivation is upon the motility apparatus itself or as a result of the phosphorylation of proteins in the plasma membrane (Noland et al, 1984) remains to be elucidated.

Through the use of the fluorescent probe Quin-2, these studies have shown that the intracellular concentration of calcium increases at the time when cAMP levels are rising, confirming the observations that a net influx of calcium accompanies capacitation in this and other species (Singh et al, 1978; Triana et al, 1980; Singh et al, 1980). Increases in the levels of this cation could provide the stimulus for the elevation of cAMP content via an action upon the adenylate cyclase system, which is known to be calcium dependant in spermatozoa (Kopf and Vacquier, 1985). Displacement of

calcium from membrane bound sites has been implicated as the mechanism by which a number of membrane active agents stimulate sperm movement (Singh et al, 1980; 1983). Such an action would lead to an increase in $[Ca^{2+}]_i$, which, in turn, could stimulate adenylate cyclase activity. The intriguing but complex inter-relationship between cAMP, calcium and sperm motility clearly requires further investigation.

As detailed above, the surface of spermatozoa undergoes changes during epididymal maturation. In a similar manner, capacitation is also associated with modifications of the plasma membrane. These alterations include the loss of surface antigenic determinants (Oliphant and Brackett, 1973; Koehler, 1976) and decapacitation factors (Chang, 1957; Eng and Oliphant, 1978), and changes in the patterns of lectin binding (Gordon et al, 1975; Talbot and Franklin, 1978; Kinsey and Koehler, 1978; Courtens and Fournier-Delpech, 1979; Schwarz and Koehler, 1979; Lewin et al, 1979; Byrd, 1981; Ahuja, 1984). Although the majority of these changes take place over the acrosomal region, and are thought to faciliate the membrane fusion events associated with the acrosome reaction and fertilization, some alterations of the flagellar membrane have also been detailed (Talbot and Chacon, 1978; Talbot and Franklin, 1978; Kinsey and Koeher, 1978; Schwartz and Koehler, 1979). These have more recently been correlated with the onset of hyperactivation in hamster spermatozoa (Ahuja, 1984), implying that a relationship exists between intracellular and surface changes in capacitating spermatozoa. The process by which forward motility protein binds to the flagellum of bovine epididymal spermatozoa, acting in concert with increasing levels of cAMP levels to facilitate the appearance of coordinated motility, may be analagous to the membrane changes demonstrated to occur in capacitating hamster spermatozoa, which coincide with both increases in cAMP levels and alterations of movement characteristics. Whether these changes reflect the increase in the permeability of the membrane to calcium is unknown.

Catecholamines and the amino acids taurine and hypotaurine have been implicated as "triggers" for the onset of hyperactivation (Cornett and

Meizel, 1978; Bavister et al, 1979; Mrnsy et al, 1979; Meizel et al, 1980; Liebfreid and Bavister, 1981, 1982). Although the mechanisms by which these agents operate have not been conclusively demonstrated, it seems likely that non-specific inhibition of lipid peroxidation accounts for a large proportion of their actions (Alvarez and Storey, 1983), although an action via elevation of cAMP levels cannot be discounted (Cornett and Meizel, 1978; Mrsny and Meizel, 1980). Furthermore, the inhibiton of hyperactivation which resulted from treatment with the beta-blocker propranolol (Cornett and Meizel, 1978), although interpreted as an implication of the involvement of adrenergic receptors in the expression of this motility pattern, may actually indicate the importance of membrane events in the induction of hyperactivated motility, as propranolol is a membrane stabilizing agent (Hong and Turner, 1982).

Spermatozoa from the caput epididymis of the hamster were found to be unable to express an increase in cAMP levels upon prolonged incubation, in contrast to the rise expressed by caudal cells. The reasons for this inability did not appear to be due to deficiences in calcium levels, ATP content, intracellular pH or responsiveness of the adenylate cyclase system. However, the observation that repetitive centrifugation caused a stimulation of caput cAMP levels, whilst depressing those of caudal cells, may indicate that the membrane changes which occur during epididymal maturation are involved in the development of the ability to express a rise in cAMP levels. In this context, the recent observation that both invertebrate and mammalian spermatozoa possess an inhibitory guanine nucleotide-binding regulatory protein (Kopf et al, 1986) may be of importance. Changes in membrane fluidity could affect the relationship between this sub-unit and the adenylate cyclase enzyme. Such alterations could account for the epididymal development of the capacity to express a rise in cAMP levels.

## Part B: Male Contraception

# Chapter 6. General Introduction.

The purpose of this chapter is to acquaint the reader with the various means which have been either proposed or employed as contraceptive agents in the male; their mode of action, efficacy and drawbacks.

A. Background. Probably the oldest method of male contraception is the practice of coitus interruptus (male withdrawal), which is mentioned in the Bible, the Talmud and the Koran (Potts and Diggory, 1983). It remained the most common single method in Europe until after World War II, and is still the leading contraceptive used in Italy (Potts, 1985). However, records extend for over 4000 years detailing the use of various pastes, gums and gels as vaginal contraceptives (Sciarra, 1979). Vasectomy was begun in the 19th century, and is now such a simple operation that six million vasectomies were performed in the United States in the 1970's (Stokes, 1980). Condoms were first described by the Italian anatomist Fallopius in 1564 (Forrest, 1984), who detailed the use of sheaths made of linen that provided protection against syphilis. Animal intestines were also employed for similar purposes. With the development of vulcanized rubber in the 1840's, the condom became the first mass-produced contraceptive aid.

The condom provides an ideal method of contraception in a promiscuous society, being effective, easily available, socially acceptable, and in addition offering the advantage of protection against venereal diseases. In a more stable relationship however, the use of condoms is frequently not acceptable to both partners. Since another potent, reversible male antifertility agent is not available, the woman is again forced to bear the contraceptive burden alone. Vasectomy may become an alternative when the family has been completed, but psychological factors and the desire to maintain potential fertility for unseen future events deter many men from this, possibly irreversible, step. The development of a male analogue to the female pill is thus required. It has been determined that 70% of American men would accept such a method if it were available (Keith et al, 1975). Attempts to develop a male contraceptive can be divided into two broad areas; those whose mechanism of action operates via some aspect of the

endocrine system in the male, and those which act directly upon the germ cells themselves.

## B. Approaches to male contraception.

### i. The Endocrine approach.

a. GnRH analogues. Spermatogenesis in the human requires the stimulatory actions of the gonadotropins secreted by the anterior pituitary; namely follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Dizerega and Sherins, 1980). The production of these hormones is, in turn, regulated by gonadotropin-releasing hormone (GnRH), which is produced by the basal hypothalamus. In view of the central role played by GnRH, analogues of this compound have been proposed as possible candidates for a male contraceptive agent. Although treatment with GnRH agonists such as buserelin (Berquist et al, 1979) was found to cause a reduction in the numbers of spermatozoa present in the ejaculate (Linde et al, 1981; Rabin et al, 1981), this action was accompanied by impotence and a loss of libido. Similar undesirable side-effects resulted from treatment with GnRH antagonists (Weinbauer et al, 1984). The reduction of testicular function caused by these reagents thus requires androgen replacement if GnRH analogues are to be made acceptable for male fertility regulation. However, such treatment in turn presents a problem in that replacement testosterone reduces the suppression of spermatogenesis, even when the replacement is delayed as long as possible (Doelle et al, 1983; Schurmeyer et al, 1984; Michel et al, 1985; Bhasin et al, 1985). Therefore, a contraceptive strategy based upon the use of GnRH analogues plus testosterone replacement would not currently appear to present a feasible means for regulating fertility in the male.

b. Androgens. The secretion of FSH and LH are regulated by a

negative feedback mechanism which operates via the gonadal steroids. It should thus be possible to suppress spermatogenesis by the exogenous administration of testosterone. The general validity of this basic concept was demonstrated almost four decades ago (Heller et al, 1950). Since then, several different forms of testosterone have been utilised in fertility studies.

Danazol (17 -pregn-4-en-20yno-(2,3-d) isoxazol-17-ol), a synthetic analogue of ethinyl testosterone, was found to cause a moderate reduction of sperm counts when administered on its own (Sherins et al, 1971; Skolgund and Paulsen, 1973), but when presented in combination with methyl testosterone, testosterone proprionate or testosterone enanthate, this compound caused a severe reduction in sperm numbers (Sherins et al, 1971; Skolgund and Paulsen, 1973; Ulstein et al, 1975). However, the relatively high doses required for these effects, and the lack of uniform response in all subjects impeded the development of danazol as a male contraceptive.

Treatment with testosterone propionate was found to induce complete and fully reversible azoospermia in those injected daily with this compound (Reddy and Rao, 1972). However, the need for high frequency intramuscular injections precluded the use of this reagent as a male contraceptive and consequently longer-acting testosterone esters were tested for male fertility control. Testosterone enanthate (TE), again administered by injection, was found to severly supress sperm production in rats (Walsh and Swerdloff, 1973; Flickinger, 1978; Kuhl et al, 1979), mice (Goldberg, 1984) and humans (Mauss et al, 1974; Steinberger and Smith 1977; Paulsen et al, 1978; Swerdloff et al, 1979). However, difficulty in achieving complete azoospermia, and the frequency of injection required (approximately one per week) due to the relatively short half life of this ester, rendered this compound unacceptable as a male contraceptive. The pharmacological properties of testosterone cypionate and cyclohexanecarboxylate are similar to those of TE (Schulte-Behrbuhl and Neischlag, 1980; Schurmeyer and Neischlag, 1984), making these derivatives no more acceptable than those previously tested. Administration of the orally effective testosterone ester,

testosterone undecanoate had only limited suppressive effects on fertility (Neischlag et al, 1978). However, studies employing 19-Nortestosterone esters, which have a longer half-life than most other testosterone esters, revealed that this group of compounds severely diminished sperm counts without adversely affecting either libido or potence (Schurmeyer et al, 1984b; Knuth et al, 1985). Further investigation of this compound (Ziporyn, 1984), and the development of other novel, long-acting, testosterone esters (Diczfalusy, 1986), such as tetosterone trans-4-n-butylcyclohexyl-carboxylate (Weinbauer et al, 1986) may herald a breakthrough in this area of male fertility control. Alternatively, the employement of new long-acting contraceptive delivery systems such as polylactic and polyglycolic microspheres (Lewis and Tice, 1984; Asch et al, 1986), may extend the duration of action of extant testosterone esters. Preliminary studies utilizing this method of drug presentation have already yielded encouraging results (Turner and Kirkpatrick, 1982). However, it should be noted that long-term use of androgens may result in liver damage and hepatic tumours (Farrell et al, 1975; Westaby et al, 1977; Falk et al, 1979). Such side-effects would presumably block the use of androgen therapy as a means to control male fertility.

Although the use of the progestogenic anti-androgen, cyproterone acetate, has been investigated as a potential male contraceptive agent (Prasad et al, 1970; Morse et al, 1973; Roy et al, 1976; Moltz et al, 1980; Wang and Yeung, 1980), the consequent drop in serum testosterone levels and the resultant loss of potency and decrease in libido rendered these agents of little more use than testosterone analogues when used at high doses. Although the use of lower concentrations of this reagent were reported to reversibly inhibit spermatogenesis without any apparent detrimental effect on libido (Petry et al, 1972), subsequent studies detailed only marginal reductions in sperm numbers (Fogh et al, 1979; Wang and Yeung, 1980). It would thus appear that cyproterone acetate is unsuitable as a male contraceptive agent.

c. Gestagens and Estrogens. The administration of gestagens is known to reduce testosterone levels , and for this reason they are used in the treatment of prostatic cancer (Robinson and Thomas, 1971; Harper et al, 1976). As such treatment also brings spermatogenesis to a halt, the possibility of developing a male contracepive based on an estrogen has been investigated (Briggs and Briggs, 1974). However, the occurence of unacceptable side effects make this avenue of research unattractive.

In the late 1950's, progesterone and several synthetic analogues of this hormone were shown to induce reversible azoospermia in men (Heller et al, 1958, 1959). Since then, several combinations of steroids have been investigated as possible male anti-fertility agents. Although combinations of 17-hydroxyprogesterone caproate with TE, medroxypregesterone acetate with TE, and levonorgestrel with testosterone have been tested (Bain et al, 1980; Foegh et al, 1980a, b), depot medroxyprogesterone acetate (DMPA) has yielded the best results so far, although due to diminished libido and potency, this compound must also be administered with testosterone replacement in the form of the testosterone esters, TE (Alvarez-Sanchez et al, 1977; Brenner et al, 1977; Frick et al, 1977a, b; Melo and Coutinho, 1977; Sanchez et al, 1979; Bain et al, 1980), testosterone propionate (Frick et al, 1977a), or testosterone cypionate (Friedl et al, 1985). However, DMPA only induced consistent azoospermia in approximately 30% of the volunteers, with the remainder exhibiting various degrees of oligozoospermia. Although on the surface this would appear to make DMPA unnattractive as a anti-fertility agent in the male, recent studies have shown that the spermatozoa which remain after treatment with DMPA plus TE are unable to fuse with and penetrate zona-free hamster ova (Wu and Aitken, unpublished observations). These findings have thus renewed the interest in this approach to male contraception.

d. Inhibin. Although, as stated above, the secretion of FSH and LH are regulated by a negative feedback mechanism which operates via the gonadal steroids, this process also appears to be controlled by a peptide of

gonadal origin. This substance, termed inhibin, causes selective supression of the secretion of FSH by the pituitary gland (Franchimont et al, 1978, 1979). In view of this ability to preferentially inhibit FSH production, which should, in turn, lead to a reduction of germ cell production, inhibin has been promoted as a potential male contraceptive agent (Franchimont et al, 1978). However, some researchers believe that the specific inhibition of FSH following inhibin administration would be unlikely to induce azoospermia, as limited spermatogenesisis is known to persist even in the absence of FSH (Matsumoto et al, 1984; Bremner and Matsumoto, 1986; Nieschlag, 1986). In contrast to these views, however, active or passive immunisation of monkeys against ovine FSH did result in azoospermia or severe oligozoospermia (Murty et al, 1979; Wickings and Neischlag, 1980), and suppression of fertility has been achieved following the administration of impure inhibin preparations to male primates (Moudgal et al, 1985). Further animal studies, preferably utilizing purified inhibin, are therefore required to examine the contraceptive possibilities of this compound. However, even if this compound does consistently induce reversible azoospermia, further difficulties will surely be encountered, such as the problems of long term administration of a polypeptide substance. Also, the cost of producing either purified inhibin or a synthetic analogue is liable to be high, as such a process would probably involve either recombinant DNA technology or peptide synthesis, in addition to separation and purification procedures.

**ii. The Direct approach.**

a. Anti-sperm antibodies. Although it was shown in the early 1930's that women injected with their husbands semen became temporally infertile (Baskin, 1932), it was not until 1954 that two investigators separately and independantly reported the existence of antibodies in the ejaculates of men whose spermatozoa exhibited the phenomena of agglutination (Rumke, 1954; Wilson, 1954). The occurence of these antibodies in semen was

subsequently correlated with an inability to penetrate cervical mucus (Fjallbrant, 1965) and to achieve pregnancy (Rumke et al, 1974; Fuchs and Alexander, 1983). The exact cause of the infertility which results from the presence of antibodies against spermatozoa appears to be due to either an impedance of cervical mucus penetration (Fjallbrant, 1965; Kremer and Jager, 1976; Alexander and Fulgham, 1978; Alexander 1981, 1984) and /or an inhibition of sperm-egg interactions (Russo and Metz, 1974; Menge and Black, 1979; Tzartos, 1979; Dor et al, 1981; Aitken et al, 1981; Bronson et al, 1983; Alexander, 1984).

Experimental studies have demonstrated that the active immunisation of adult males with antigens derived from intact or extracted spermatozoa can induce infertility in both rats and rabbits (Tung and Woodroffe, 1978; Lipscomb et al, 1979). Such treatment results in the development over a 2-6 week period of aspermatogenic autoimmune orchitis, characterized by the appearance of oedema in the intertubular spaces of the testis, and ultimately by the complete sloughing of the germinal epithelium. These lesions appear to be induced predominantly by a process of cell mediated immunity, as thymectomy of adult mice prevents the induction of autoimmune orchitis (Vojtiskova and Pokorna, 1964). However, due to the pain and inflammation, as well as doubts over the reliability of such treatment, it is unlikely that the intentional induction of auto-immune orchitis for contraceptive purposes would be acceptable.

A more realistic approach to an immunological male contraceptive is to identify sperm antigens which are capable of inducing a sustained form of humoral immunity. In this context, encouraging results have been obtained following the immunization of female baboons with the sperm specific isozyme of lactate dehydrogenase (LDH-C4) isolated from mouse spermatozoa (Goldberg et al, 1981). Furthermore, monoclonal antibodies raised against certain components of the sperm membrane have been shown to block fertilization both in vitro and in vivo (Saling et al, 1983; Naz et al, 1984; O'Rand and Irons, 1984). However, worries exist over the possibilty that anti-sperm antibodies may react with early embryos (Menge

and Fleming, 1978; Koyoma et al, 1974), could exert an immunosuppressive effect, or increase susceptibility to arterial disease (Aitken, 1982). It would thus appear that the development of a vaccine against spermatozoa will require a great deal of further basic research before this method of contraception becomes a viable alternative to the means presently available. The identification of a specific antigen is of paramount importance, although combinations of anti-bodies, or anti-bodies which interfere with, rather than directly block the cell recognition events of fertilization may serve a contraceptive role just as efficiently.

b. Alkylating agents. In the late 1950's, several potent alkylating agents (tretamine, triethylenephosphoramide, hexamethylphosphoramide and hexamethylmelamine) were found to induce reversible infertility in male rats (Jackson and Bock, 1955; Jackson et al, 1959). The antifertility effects of these compounds apparently acted through effects on sperm DNA, causing arrest of embryonic development. The toxicity of these compounds coupled with fears for the production of genetically defective foetuses caused further study of these compounds to be abandoned. However, bulsuphan, a less cytotoxic alkylating agent than those previously tested, was also found to exhibit antifertility effects in male rats (Jackson et al, 1961). This compound appeared to interfere specifically with the developing spermatogonia, and thus the onset of infertility was not apparent for a long period. Attempts to synthesise more potent and less toxic derivatives of these compounds have so far been unsuccessful (Jackson et al, 1961; Cooper and Jackson, 1970; Hirsch et al, 1981).

c. 5-Thio-D-glucose. This chemical analogue of D-glucose was found to cause complete, but reversible, inhibition of sperm development within three weeks of administration to mice, without affecting libido (Zysk et al, 1975). Similar effects were demonstrated in rats (Homm et al, 1977). Although the mechanism of action of this compound has not been elucidated, it may interfere with testicular carbohydrate metabolism.

However, persistent infertility after cessation of drug treatment (Homm et al, 1977; Lobl and Porteus, 1978) prevented the development of this compound as a male contraceptive agent

d. α-chlorohydrin. Experiments performed in the late 1960's revealed several orally active agents which had rapidly acting antifertility effects upon male rats (Ericsson and Youngdale, 1970). Of these, the most studied has been 3-chloro-1,2-propranediol, perhaps better known as α-chlorohydrin. This compound is known to cause reversible infertility in males of several species (Ericsson and Baker, 1970; Kirton et al, 1970; Vickery et al, 1974; Ford et al, 1979), and also to directly inhibit sperm motility (Hommonai et al, 1975; Mohri et al, 1975; Ford et al, 1979; Paz and Homonnai, 1982). α-chlorohydrin's antifertility effects are thought to be mediated via the glycolytic enzymes in spermatozoa, especially glyceraldehyde-3-phosphate dehydrogenase (Mohri et al, 1975; Brown-Woodman et al, 1978; Ford and Harrison, 1980). However, this compound will not inhibit glyceraldehyde-3-phosphate dehydrogenase activity directly, and it is thought that an active metabolite is formed within spermatozoa, most probably S-α-chlorolactaldehyde, which has the same configuration as R-glyceraldehyde-3-phosphate, the substrate for glyceraldehyde-3-phosphate dehydrogenase (Ford and Waites, 1986). However, an inhibitory action directed solely at this enzyme would not be expected to completely account for the antifertility effects of α-chlorohydrin, as spermatozoa could utilize lactate or pyruvate as alternative substrates. Indeed, further investigation has revealed that the inhibition of glyceraldehyde-3-phosphate dehydrogenase stimulates the active dissipation of ATP through "futile substrate cycling" within the glycolytic pathway (Ford and Harrison, 1985), leading to rapid cell death. Interestingly, the disruption of fetility which resulted from α-chlorohydrin treatment was not accompanied by a reduction of motility or sperm numbers (Ericsson and Baker, 1970; Kirton et al, 1970) implying that the antifertility

effects of this compound may operate via a more subtle disruption of sperm function than by suppression of metabolism.

In view of the toxicity of α-chlorohydrin (Jackson et al, 1977), attempts were made to develop more potent and less toxic analogues. Although antifertility agents with reduced levels of toxicity were produced (Hirsch et al, 1975; James et al, 1978), none of these were deemed to be sufficiently devoid of adverse side-effects to warrant further development.

However, the close structural similarity seen to exist between α-chlorohydrin and the metabolites of glucose stimulated an investigation of the antifertility effects of the chloro-derivatives of six carbon sugars. From the many sugars tested, it appears that the substitution of a chlorine (Ford and Waites, 1978), or fluorine (Ford, 1982a) group on the sixth carbon atom is required for the expression of antifertility activity. Of these compounds, the most potent is 6-deoxy-6-chloroglucose (Ford and Waites, 1978; Heifeld et al, 1979; Warren et al, 1979). The readily reversible antifertility effects of this reagent were thought to be due to an impairment of the oxidation of glucose in spermatozoa (Ford et al, 1981; Ford and Harrison, 1981). Although this avenue of male fertility control appeared promising, the demonstration that the central nervous system of marmosets, mice and rats was adversely affected by this compound (Jacobs and Duchin, 1980; Jacobs and Ford, 1981; Ford and Waites, 1981; Ford, 1982b) led to a decline in the interest in these reagents as possible male contraceptive agents

e. Gossypol. Gossypol (1,1$^1$,6,6$^1$,7,7$^1$-hexahydroxy-5,5$^1$-diisopropyl-3,3$^1$-dimethyl (2,2$^1$-binapthalene)-8,8$^1$-dicarboxaldehyde) is a lipid soluble, polyphenolic yellowish pigment isolated from the cottonseed plant (Family Malavaciae, Genus Gossypium)(Edwards, 1958). In 1957, it was reported that Wang village in Jiangsu, China had not had a single childbirth for as long as ten years (Liu, 1957). It was subsequently found that during this time the villagers had switched, for economic reasons, from using soybean oil to crude cottonseed oil in the preparation of food. In view of this, Liu (1957)

suggested that gossypol, the biologically active substance in cottonseed, may cause infertility in females.

It was not until 1978 that gossypol was identified as being a male contraceptive agent. In this year it was announced that 4000 healthy Chinese men had been administered gossypol for a period of 6 months, and that 99.98% of these had been rendered infertile on the basis of conventional semen examination (National Coordinating Group on Male Antifertility Agents, 1978). The oral administration of this drug was conducted in two phases. The first, an inductive phase of 20mg/day, was continued until oligozoospermia was achieved (defined as a sperm count of below 4 million/ml). In most cases this took about two months. The second, maintenance phase, consisted of single doses of 150-220 mg/month. This treatment was reported to cause nuclear vacuolation and swelling of the spermatozoa, whilst prolonged dosage caused almost complete exfoliation of the germinal epithelium, with the exceptions of the Sertoli cells and the spermatogonia (National Coordinating Group on Male Antifertility Agents, 1978). This group further reported similar lesions and morphological effects in laboratory animals treated with gossypol. It has since been shown that in addition to the human, gossypol is effective in inducing infertility in rats and hamsters (Chang et al, 1980; Hadley et al, 1981; Hahn et al, 1981; Qixian and Yuying, 1981; Waller et al, 1981; Weinbauer et al, 1982), although its antifertility actions are less pronounced in monkeys and mice (Hahn et al, 1981; Shandilya et al, 1982).

A variety of mechanisms have been proposed for gossypol's antifertility effects, including the disruption of testosterone biosynthesis (Lin et al, 1981; Hadley et al, 1981), inhibition of germ cell differentiation (National Coordinating Group on Male Antifertility Agents, 1978; Hadley et al, 1981; Xue, 1981), disruption of Sertoli-Sertoli junctions, leading to a breakdown of the blood-testis barrier (Pelletier and Friend, 1980) and the induction of gonadal prostaglandin biosynthesis (Qian, 1981). In addition to its testicular effects, gossypol has been shown to have a direct inhibitory action upon the motility of mature spermatozoa both in vitro and in vivo

(Waller et al, 1980; Poso et al, 1980; Ridley and Blasco, 1981; Tso and Lee, 1981; Cameron et al, 1982; Aitken et al, 1983; Chongthammakun et al, 1986). The mechanism by which gossypol disrupts motility may involve the inhibition of glycolysis (Stephens et al, 1983; Wichmann et al, 1983), the uncoupling of oxidative phosphorylation at the mitochondrial level (Abou-Donai MB, Dieckert JW, 1974; Poso et al, 1980; Xue, 1981; Tso and Lee, 1982a), or the specific inhibition of the gonadal form of lactate dehydrogenase, LDH-X (Tso and Lee, 1981, 1982b).

Although gossypol is an effective anti-fertility agent, reservations about its use as a male contraceptive have arisen because of reports of possible toxicity, side-effects and incomplete reversibility of spermatogenesis after drug withdrawal (Prasad and Diczfalusy, 1982). However, in view of the relatively small doses required to directly inhibit sperm motility (Poso et al, 1980; Ridley and Blasco, 1981; Stephens et al, 1983; Waller et al, 1983), it has been suggested that gossypol has potential as a topical, vaginal contraceptive (Waller et al, 1980; Poso et al, 1980; Tso and Lee, 1981; Cameron et al, 1982; Waller et al, 1983; Ratsula et al, 1983). Further investigation of the specific anti-fertility mechanism of this drug may allow the distillation of a potent male contraceptive agent devoid of deleterious side effects.

f. Sulphasalazine. Sulphasalazine (4-pyridyl-(2)-aminosulphonyl-3-carboxy-41-hydroxybenzol - also known as Salicylazosulphapyridine, Azulfidine and Salazopyrin) is an anti-inflammatory drug which has been employed in the management of inflammatory bowel disease since the early 1940's (Baron et al, 1962). Although originally developed and introduced as a therapy for rheumatoid arthritis (Svartz, 1942), Its use is now established for the treatment of ulcerative colitis, and more recently it has been used to combat the effects of colonic Crohn's disease (Summers et al, 1979). This drug had been in constant daily use for almost forty years before two groups separately and independantly reported an association between sulphasalazine therapy and male infertility (Levi et al, 1979; Toth, 1979).

Chronic administration of the drug was reported to cause a reduction in sperm density and sperm motility, whilst increasing the numbers of morphologically abnormal spermatozoa present in the semen. Several independant studies have subsequently confirmed and enlarged upon these original observations (Grieve, 1979; Traub et al, 1979; Birnie et al, 1981; Toovey et al, 1981; Mudge, 1982).

An analysis of the onset of seminal abnormalities in patients taking sulphasalazine indicated that sperm motility was impaired before density and morphology were affected (Toovey et al, 1981). Hence, seven men treated for less than 2 months presented samples in which only 30 ± 1.5 % of the spermatozoa were progressively motile , significantly different from the normal range, which has a lower limit of 40%. In contrast, the concentration of spermatozoa in these semen samples (82.5 ± 21.9 x $10^6$/ml) was within the normal range (>40 x $10^6$/ml), as was the sperm morphology (70% normal forms). However, when the duration of sulphasalazine treatment was extended for more then two months in 17 patients, there was a significant reduction in both the concentration of spermatozoa (20.9 ± 4.2 x $10^6$/ml) and in percentage motility (29.0 ± 4.2% progressively motile). Morphology was also affected significantly after 2 months of sulphasalazine therapy, with 39 ± 4.5% abnormal forms being present. All three seminal parameters measured tended to improve within two months of sulphasalazine withdrawal, although none of these changes was statistically significant. Thus, two months after the cessation of treatment, 11 out of 14 patients possessed normal concentrations of spermatozoa in the ejaculates (53.6 ± 6.9 x $10^6$/ml) and exhibited some improvement in sperm motility (45.1 ± 2.5% progressively motile), although defects in sperm morphology were still evident.

Data from a number of centres indicated that upon cessation of sulphasalazine therapy, normal fertility returns to patients before any seminal characteristics have reverted to normal levels (Toovey et al, 1981; Cann and Holdsworth, 1984; Cosentino et al, 1984). It would therefore seem

that the primary antifertility action of this drug is occurring late in spermatogenesis or during the epididymal maturation of spermatozoa. These findings also indicate that the actual mechanism by which sulphasalazine renders spermatozoa infertile may be by a much more subtle alteration of sperm function than the gross changes detected in the conventional semen profile. In contrast to the changes in both sperm function and the semen profile, the circulating levels of LH, FSH, testosterone, 5-α-dihydrotestosterone and prolactin show no changes before, during or after sulphasalazine treatment (Toovey et al, 1981; Cosentino et al, 1984a; Chodorge et al, 1986), suggesting that the antifertility action of this drug does not have an overt endocrinological basis. However, the exact mode of action of this drug remains unknown.

g. Spermicides. These agents act upon spermatozoa as they pass through the female genital tract. The first commercial spermicide was developed by an English pharmacist in 1885, who patented the Rendell pessary. This contained soluble cocoa butter and quinine sulphate (Sciarra, 1979). Since then, many different compounds have been utilized as spermicides, including mercury, quinine and boric acid. In the early 1950's, the nonionic detergent nonoxynol-9 (nonylphenoxypolyethyleneoxyethanol) was introduced, and this agent remains the spermicide in widespread use at the moment. It is used on the spermicidal condom and in spermicidal foam, creams, gels, suppositories and sponges. The basis of this compound's spermicidal activity resides in its ability to solubilise the sperm plasma membrane, causing rapid immobilization and cell death (Schill and Wolf, 1981; Wilborn et al, 1983).

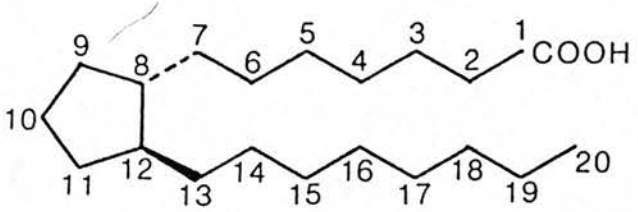
As nonoxynol-9 is a surfactant, it may have problems contacting spermatozoa, particularly when they are entrapped in the coagulum, or if they rapidly enter cervical mucus (Sharman et al, 1986; Zanaveld et al, 1986). Thus, several other compounds have been investigated as potential vaginal contraceptives. The spermicidal agent chlorohexidine overcomes some of the problems of nonoxynol-9 by affecting sperm motility during its

passage through cervical mucus (Sharman et al, 1986). However, this compound is known to cause vaginal irritation in some people (E Chantler, personal communication). Further development of this compound as a spermicide awaits the results of toxicological studies.

Inhibitors of acrosin, implicated as the enzyme responsible for sperm penetration of the zona pellucida (Stambaugh and Buckley, 1969), and hyaluronidase, associated with the penetration of the cumulus oophorus (McClean and Rowlands, 1942), will inhibit fertilization (Zanaveld et al, 1971; Meizel and Lui, 1976; Reddy et al, 1980; Burck and Zimmerman, 1980). Indeed tetradecyl sodium sulphate inhibits both of these enzymes (Zimmerman et al, 1983). However, the acrosin inhibitors aryl 4-guanidinobenzoates have attracted the most attention, with several of these compounds exhibiting greater potency than nonoxynol-9 (Zanaveld et al, 1986). Further clinical testing will reveal whether these compounds present viable alternatives to nonoxynol-9.

The widely used beta-blocking drug, propranolol, is known to inhibit human sperm motility (Peterson and Freund, 1973, 1975; De Turner et al, 1978; Zipper et al, 1982). The basis of this compound's spermicidal action is thought to depend upon its local anaesthetic properties, rather then its beta-blocking ability, as the purified D-isomer has been found to be as effective as the racemic mixture in supressing sperm motility, despite the fact that D-propranolol does not possess the ability to block beta-receptors (Peterson and Freund, 1973, Hong et al, 1981). This compound has also been proposed as an alternative vaginal contraceptive (Chijioke et al, 1986).

# Chapter 7. Investigation of the mechanisms of action of sulphasalazine and gossypol.




NUMBERING OF CARBON ATOMS ON PROSTAGLANDIN SKELETON

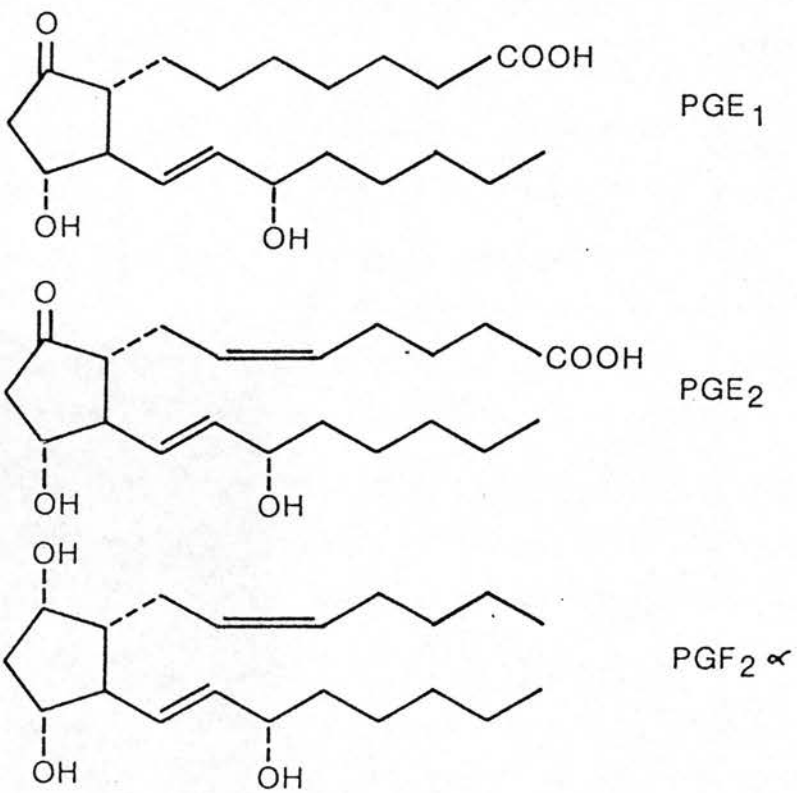



Figure 45. (a) The numbering of carbon atoms on the prostaglandin skeleton. (b) The different molecular structure of the classical prostaglandins. Redrawn from Ramwell et al (1980).

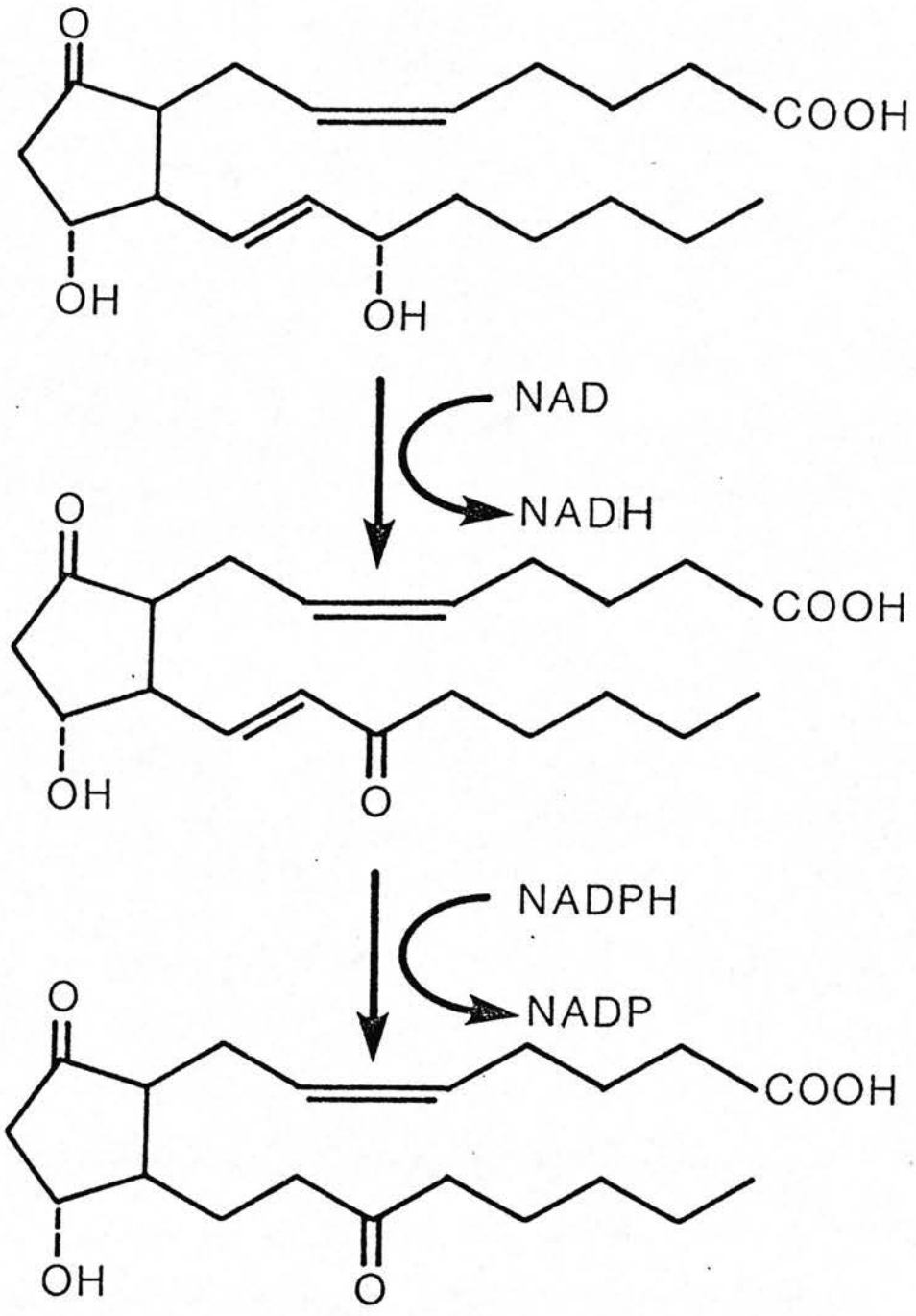



Figure 46. The initial catabolism of $PGE_2$. The oxidation of the hydroxyl group of carbon 15 to a keto group is catalysed by 15-hydroxyprostaglandin dehydrogenase (15-PGDH), whilst the reduction of the double bond between carbons 13 and 14 to a single bond is catalysed by 15-keto-prostaglandin-13,14-reductase (13-PGR). Redrawn from Ramwell et al (1980).

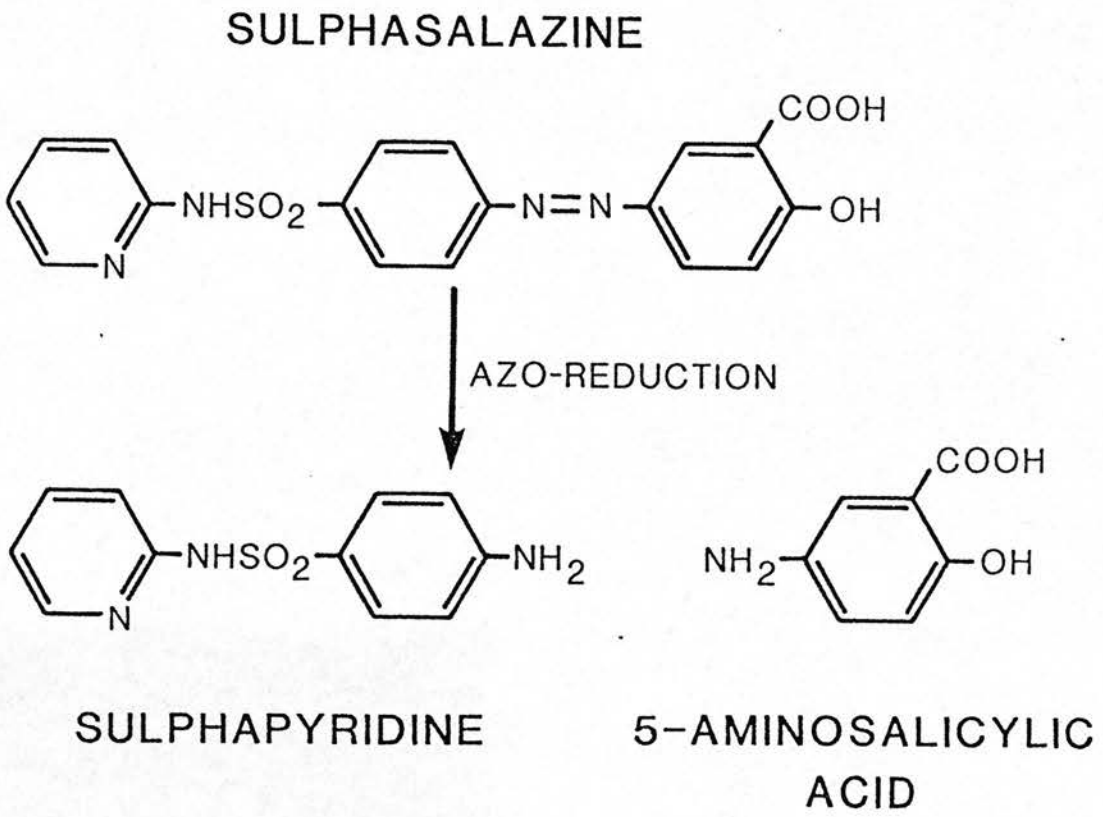



Figure 47. The azo-reduction of sulphasalzine to sulphapyridine and 5-aminosalicylic acid. Redrawn from Hoult and Moore (1980).

## A. Introduction.

The mechanism by which sulphasalazine causes infertility is unknown. However, investigations into the mechanisms by which this drug combats the effects of ulcerative colitis have shown that it is a potent inhibitor of prostaglandin metabolism (Hoult and Moore, 1980). Prostaglandins (PG's) are cyclic derivatives of certain unsaturated fatty acids with 20 carbon atoms (Fig 45). They are found in many animal tissues and have a variety of profound hormone-like, physiologic and pharmacologic properties. PG levels within tissues are governed by the rates of their production and degradation. They are not stored, and therefore their action within a certain organ is controlled as much by metabolism as synthesis. The initial catabolism of PGs involves oxidation of the hydroxyl group of carbon 15 to a keto group, then reduction of the double bond between carbons 13 and 14 to a single bond (Ramwell et al, 1980 - Fig 46). The primary enzymes involved in this metabolic pathway are 15-hydroxyprostaglandin dehydrogenase (15-PGDH), and 15-keto-prostaglandin-13,14-reductase (13-PGR). Sulphasalazine is a potent inhibitor of the initial catabolic step, catalysed by 15-PGDH, but not of the second step, catalysed by 13-PGR (Moore et al, 1978; Hoult and Moore, 1978, 1980). Inactivation of PG breakdown is postulated to invoke the cytoprotective and anti-ulcerative effects of PG's in the colonic mucosa (Hoult et al, 1979; Hoult and Moore, 1980).

Of orally ingested sulphasalazine, approximately 33% is absorbed in the small intestine, whence it undergoes entero-hepatic circulation. Acetylated and free sulphasalazine subsequently appear in the urine. The sulphasalazine that passes to the large bowel is azo-reduced by bacterial action to form sulphapyridine and 5-aminosalicylic acid (Das et al, 1973a, b - Fig 47). Neither of these metabolites has been found to inhibit 15-PGDH activity in a manner similar to the parent molecule (Hoult and Moore, 1980). Since the influence of sulphasalazine upon ulcerative colitis is thought to involve the inhibition of prostaglandin catabolism, a rational approach to the

investigation of its antifertility action was to examine the role that prostaglandins play in the regulation of male reproductive function. Although very little is known about the levels of PGs present in the human testis and epididymis, the endocrine functions of both these organs have been shown to include PG production (Buhrley and Ellis, 1975; Gerozissis and Dray, 1977). Furthermore, PGs have been shown to be present in both the testis (Carpenter and Wiseman, 1970) and epididymis (Gerozissis and Dray, 1977) of the rat, and PG metabolising enzymes have also been located in the reproductive tract of this animal (Nakano and Prancan, 1971; Nakano et al, 1971; Ohuo-Obasiolu, 1983). PGs are known to have stimulatory effects on both seminiferous and epididymal tubule contractions (Hunt and Nicholson, 1972; Buhrley and Ellis, 1975; Poulos et al, 1975; Abbatiello et al, 1976; Farr and Ellis, 1980), and are thought to bind to both human (Mercado et al, 1978), and rabbit (Bartoszewitcz et al, 1975) spermatozoa. A role for PG's in the maturation of spermatozoa in the epididymis has been postulated (Glover and Nicander, 1971; Johnson and Ellis, 1977), and they are known to have a profound effect upon the fertilizing potential of human spermatozoa in vitro (Aitken and Kelly, 1985). In addition, the exogenous administration of PGs has been reported both to suppress spermatogenesis and inhibit epididymal sperm motility in rats (Ericsson, 1973). In light of these observations, it would seem reasonable to suppose that PGs are involved in some way in the regulation of male reproductive function, and that sulphasalazine and possibly gossypol exert their antifertility effects through the disruption of PG catabolism. This study therefore investigates this possibility by measuring the activity of the 15-PGDH enzyme, as well as its regulation by sulphasalazine and gossypol, in the male reproductive tract of the rat and and the human.

## B. Materials and Methods.

i. Reagents. 13,14-dihydro-15-keto [5,6,8,11,12,14(n)-$^3$H] $PGE_2$ (80Ci/mmol) was purchased from the radiochemical centre (Amersham, England). Authentic PGs and PG metabolites were the gift of Dr J Pike (Upjohn Co, Kalamazoo, MI). Cofactors were obtained from Sigma (Poole, England). Sulphasalazine, sulphapyridine and 5-aminosalicylic acid were purchased from Pharmacia (Uppsala, Sweden). (±) Gossypol acetic acid was obtained from the Beijing Family Planning Institute (Beijing, China). The (+) and (-) enantiomers of gossypol were gifts from Dr S Matlin (City University, London). An antiserum specific for the methoximated derivatives of $PGE_2$ metabolites was a gift from Dr RW Kelly (MRC Reproductive Biology Unit, Edinburgh). Solvents (Analar grade) were purchased from BDH (Glasgow).

ii. Tissue preparation. Human testicular and epididymal tissue was removed from the cadavers of whole organ donors in the post-mortem room of the Royal Infirmary of Edinburgh. Once excised, the tissue was placed on ice for the short journey from the post-mortem room to the laboratory. Rats (Rattus norvegicus L) CD strain (Sprague Dawley), housed in the MRC Centre for Reproductive Biology's animal house under 12h daylight, with free access to food and water, were suffocated with $CO_2$ and killed by cervical dislocation Tissue from both species were divided into testicular, caput (rat, site 1-3 - Hinton et al, 1979a; human, region 1b-1d - Bedford et al, 1973), corpus (rat, site 4-5 - Hinton et al, 1979a; human, region 2a-4a - Bedford et al, 1973), or caudal (rat, site 6-7 - Hinton et al, 1979a; human, region 4b-5 - Bedford et al, 1973) epididymal regions. These were then weighed, placed in ice-cold 0.05M tris/glycerol buffer, pH 7.4, and homogenised in a polytron homogeniser (Brinkmann Instruments, Westbury, New York) at full speed for 15 seconds. Cell debris was removed by centrifugation at 1500g for 10 mins at 4°C. The supernatant was then removed and stored at a tissue concentration of 100mg/ml at -18°C. The

concentration of protein present in these tissue preparations was determined using the method of Lowry et al (1951).

iii. Incubation conditions. Prior to use, each tissue extract was thawed and diluted with ice-cold tris/glycerol buffer to a tissue concentration of 10mg/ml. 1.0ml of this solution was then pre-incubated in a shaking water bath at 35°C for 5 mins with 1mM NAD+ (added in 40µl of tris buffer) and, when appropriate, the test concentration of drug. After pre-incubation, 5µM $PGE_2$ was added as substrate in 20µl of ethanol/tris (4:1). Enzyme characteristics were determined by incubation in the presence of varying concentrations of substrate.

After a final incubation period of 15mins, the reaction was terminated by the addition of 1ml of methoxymating solution (0.12mM methoxyamine hydrochloride in 1.0M sodium acetate, pH 5.2). Methoxymation of the prostaglandins present was achieved by heating the incubation at 60°C for 30mins.

iv. Derivatisation of prostaglandins. PGs and their metabolites will not remain stable in aqueous solution. 13,14-dihydro-15-keto-$PGE_2$ ($EM_2$) undergoes non-enzymic conversion to its bicyclic derivative, 13,14-dihydro-15-keto-11ß,16 -cyclo-$PGE_2$ via the intermediate 13,14-dihydro-15-keto-$PGA_2$. These compounds comigrate upon thin layer chromatography and are collectively termed $EM_3$. To avoid these and other non-enzymic changes, measurements of the metabolites of PGs were performed upon their methyl-oxime derivatives. Authentic PGs and their metabolites were derivatised using the method of Kelly and Abel (1983). Antisera specific for $PGEM_2$ was raised against its methoxymated derivative coupled to human serum albumin. PGs and their metabolites formed in incubations were derivatised by the addition of methoxymating solution (see above). This process stopped any enzymic reactions and furthermore prevented the occurrence of non-enzymic destabilization.

v. Determination of 15-PGDH activity. The activity of this enzyme in different incubations was estimated by measuring the production of the first metabolite of $PGE_2$ catabolism, namely 15-keto $PGE_2$ ($PGEM_1$). These measurements were performed using the radioimmunoassay procedure described by Kelly et al (1986). The conversion of $PGEM_1$ to $PGEM_2$ was prevented by cryostorage of the tissue extract, which denatures the co-factor (NADPH) required for this step in the metabolic pathway (Abel and Kelly, 1983). Furthermore, the sole exogenous co-factor added to the incubation mixtures was NAD+, which only catalyses the conversion of $PGE_2$ to $PGEM_1$. For the assay of $PGE_2$ metabolites, 0.1ml (0.01μCi) of $^3H$ $PGEM_1$ in assay buffer (tris 0.05mol/l, pH7.4, with 0.05% sodium azide and 0.03% EDTA, plus 5% bovine gamma globulin) and 0.1ml of either unlabelled methoxymated $EM_1$ (4 - 2048 pg), or an aliquot from an incubation made to 0.1ml with assay buffer, were incubated with antisera specific for $EM_1$ at a dilution of 1:16000, in a 2ml capacity assay tube. Under these conditions, approximately 35% of tritiated PG was bound in the absence of unlabelled PG. The tubes were vortexed thoroughly and incubated for 1 h at 37°C, followed by overnight storage at 4°C, allowing adequate time for equilibration. The free fraction was then separated from the fraction bound to the antibody using polyethylene glycol (PEG) with a molecular weight of approximately 6000. 0.8ml PEG (300mg/ml in phosphate buffered saline, pH 7.0) was added to each tube. The mixture was then vortexed and centrifuged at 1000g for 10 mins. The supernatant was removed and the pellet resuspended in 1.0ml of cumene based scintillation fluid (Optiphase RIA, Fisons, Crawley, England) using a multitube vortexer. The radioactivity present in each tube was counted on an LKB liquid scintillation counter. Standard curve plotting and analysis of unknowns was performed on an apple benchtop computer linked to a Texas Instruments printer, pre-programmed to analyse radioimmunoassay data and perform spline fitting.

The antisera crossreacted with $PGE_1$, $PGE_2$, $PGFM_2$ (13-14-dihydro-15-keto $PGF_{2\alpha}$) at levels less than 1%, but cross reacted 100% with $PGEM_1$

and $PGEM_2$. All solvents and chemicals used in the incubations were tested for cross-reaction with the antisera. None inhibited or cross-reacted at the concentrations used in these experiments. The inter- and intra-assay coefficients of variation were 8.4% and 3.5% respectively.

vi. Statistical analysis. The statistical significance of the results was assessed using analysis of variance (Winer, 1971). Differences between the controls and various drug treatments were evaluated using Dunnetts procedure (Winer, 1971).

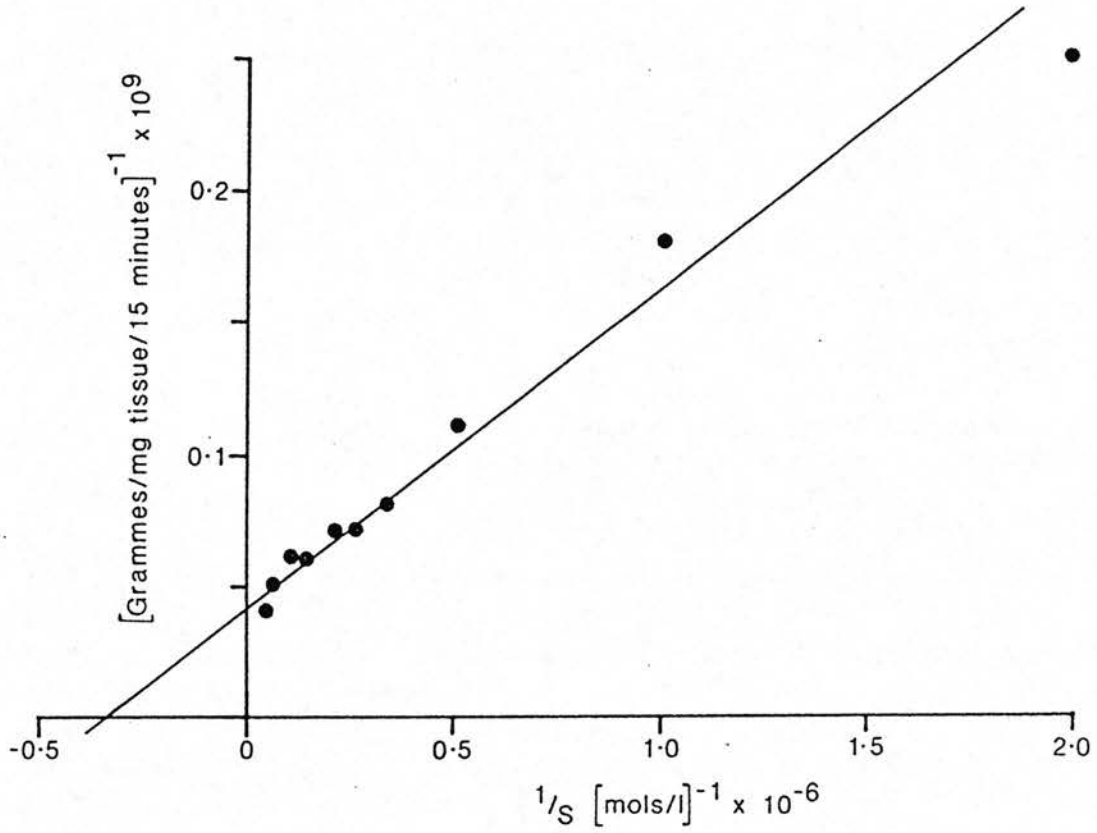



Figure 48. A Lineweaver-Burke plot of the effect of increasing substrate concentration on the conversion of $PGE_2$ to 15-keto $PGE_2$ by the NAD+ dependant 15-hydroxy prostaglandin dehydrogenase from the rat epididymis. The line of best fit was obtained using weighted regression analysis.

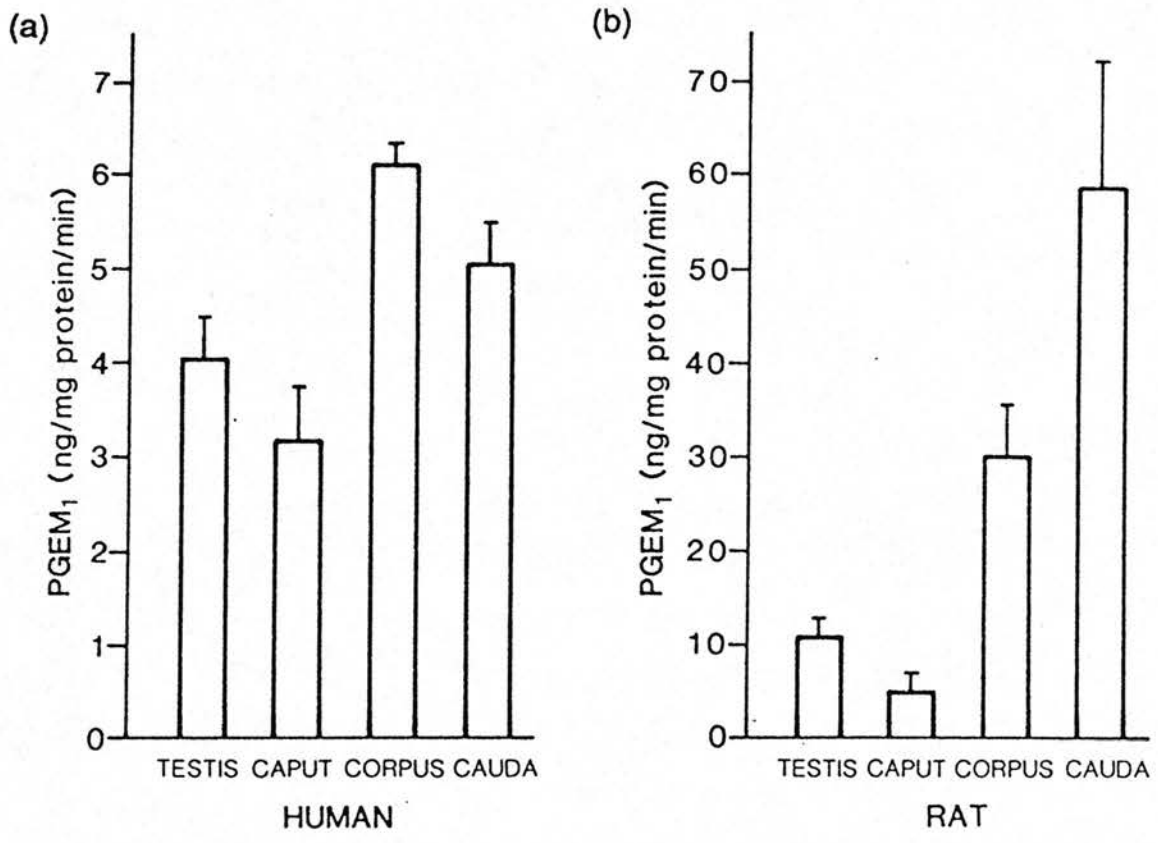



Figure 49. 15-PGDH activity in the testis and caput, corpus and cauda epididymides from the human (a) and rat (b). Values shown are mean ± sem, n=6.

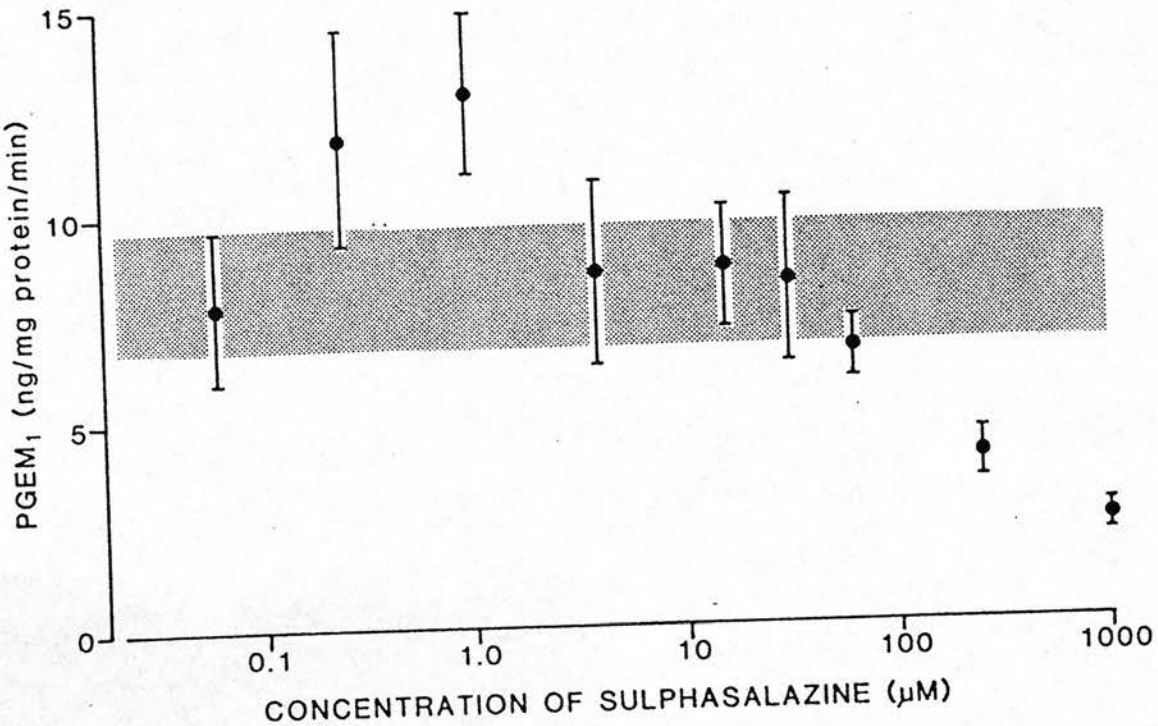



Figure 50. Dose response curve for the effects of sulphasalazine on rat epididymal 15-PGDH activity. Values shown are mean ± sem, n=6.

## C. Results.

i. Enzyme characterisitics. The characteristics of the enzyme are shown as a Lineweaver-Burke plot (Fig 48). The Km value calculated from this graph is 2.94 x $10^{-6}$mol/l, which compares with a value of 2.8 x $10^{-6}$mol/l determined for the 15-PGDH enzyme from human myometrial tissue (Abel and Kelly, 1983), indicating that the same enzyme is present in both studies.

ii. Basal enzyme levels. Appreciable levels of 15-PGDH activity were measured in the reproductive tracts of both the rat and the human. In the latter, the 15-PGDH activity (measured in terms of amount of first metabolite of PG metabolism produced per mg protein per minute) was 3.90 ± 0.32 ng/mg/min in the testis and 3.7 ± 0.38 ng/mg/min in the caput region of the epididymis. Significantly ($P<0.01$) higher levels of enzyme activity were detected in the more distal regions of the epididymis, giving 7.39 ± 0.50 ng/mg/min in the corpus and 6.34 ± 0.52 ng/mg/min in the cauda (Fig 49a). In the rat, the activity of the 15-PGDH enzyme in all regions of the reproductive tract was significantly ($P<0.01$) greater than in the human, although the relative distribution was similar. Hence in the rat testis and caput epididymis, the levels were 18.7 ± 2.4 ng/mg/min and 7.0 ± 1.5 ng/mg/min respectively, rising significantly ($P<0.01$) to 37.8 ± 5.0 and 67.9 ± 7.3 ng/mg/min in the corpus and cauda (Fig 49 b).

### iii. Enzyme response to drug treatment.

a. Sulphasalazine and its metabolites. The effects of sulphasalazine and its metabolites, sulphapyridine and 5-amino salicylate, on 15-PGDH activity was assessed using enzyme obtained from homogenates of the whole rat epididymis. A dose-dependant study revealed a biphasic response to treatment with sulphasalazine, with a stimulation of enzyme activity occurring at low levels, around 0.5 - 1.0 µM, followed by inhibition at doses in excess of 100 µM (Fig 50). The stimulation of enzyme activity was

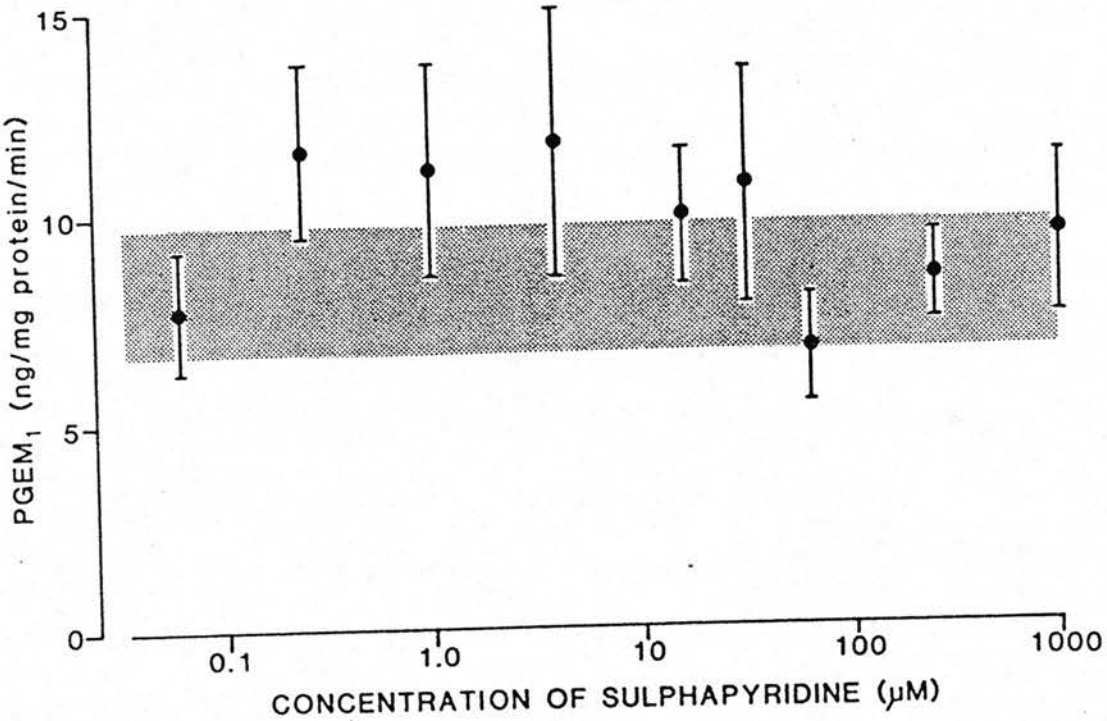



Figure 51. Dose response curve for the effects of sulphapyridine on rat epididymal 15-PGDH activity. Values shown are mean ± sem, n=6.

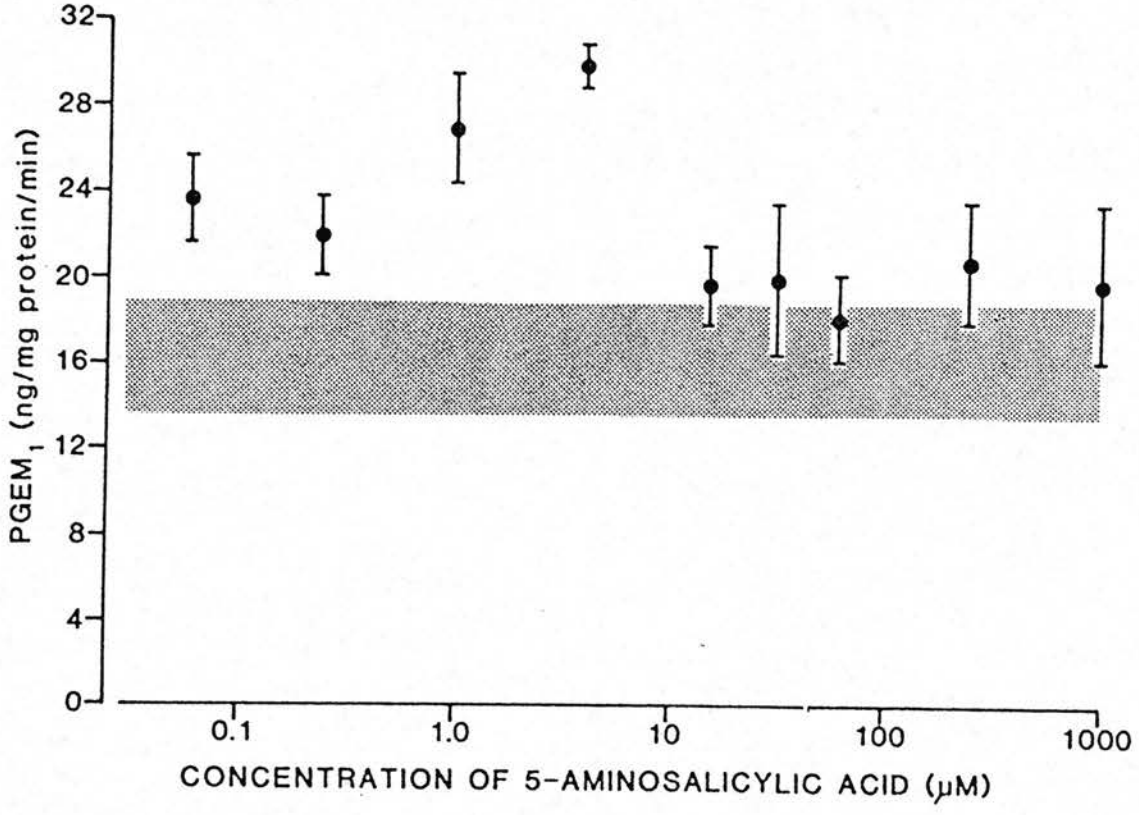



Figure 52. Dose response curve for the effects of 5-aminosalicylate on rat epididymal 15-PGDH activity. Values shown are mean ± sem, n=6.

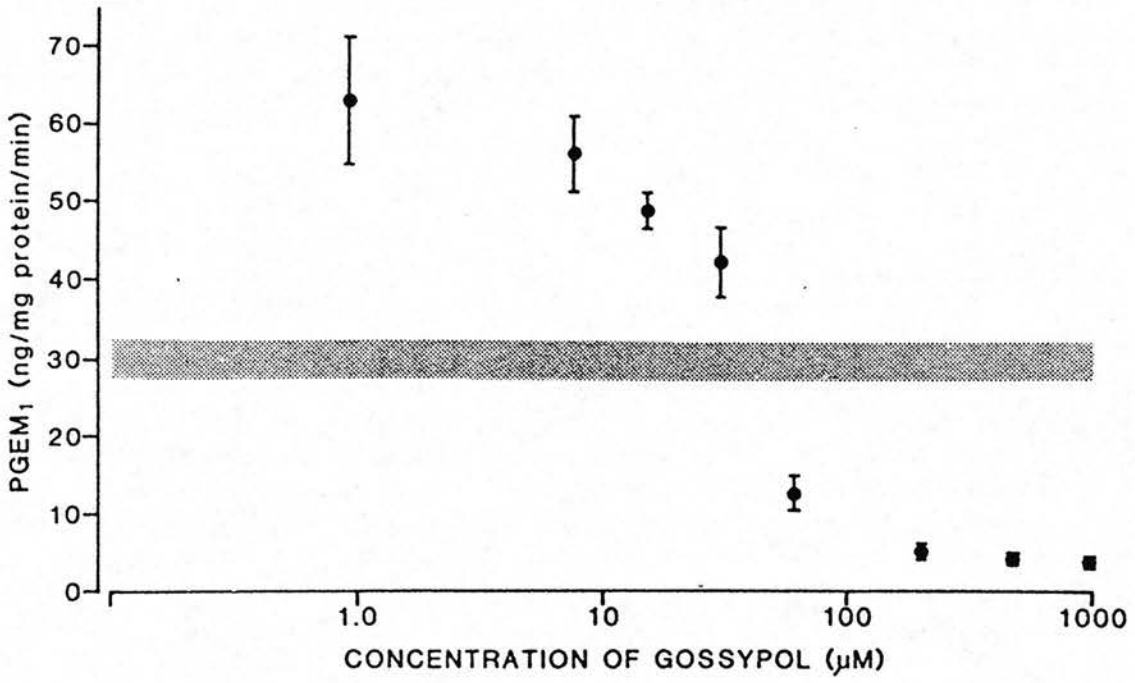



Figure 53 Dose response curve for the effects of racemic gossypol on rat epididymal 15-PGDH activity. Values shown are mean ± sem, n=6.

| TREATMENT | ACTIVITY (ng/mg/min) |
|---|---|
| Control | 7.17 ± 0.56 |
| (-) Gossypol 0.5μM | 9.71 ± 0.92* |
| (-) Gossypol 1.0μM | 8.05 ± 0.58 |
| (+) Gossypol 0.5μM | 7.45 ± 0.52 |
| (+) Gossypol 1.0μM | 8.80 ± 0.62 |

* F=4.52: P<0.05 [(-) gossypol vs control]
Dunnetts t=2.9: P<0.01 (0.5μM vs control)

Table 1. Influence of the resolved enantiomers of gossypol on rat epididymal 15-PGDH activity. Values shown are mean ± sem, n=8.

found to be statistically significant ($P<0.05$) and to represent a 30.0 ± 2.3% increase over control levels at a dose of 0.5μM. Sulphapyridine also tended to increase the activity of this enzyme over a wide range of doses, although in this case, the stimulation was not statistically significant (Fig 51). The second metabolite of sulphasalazine, 5-amino salicylate, did induce a significant increase in the activity of this enzyme, equivalent to a rise of 64.2 ± 4.2% over control levels at a dose of 1μM (Fig 52).

b. Gossypol. As shown in Fig 53, gossypol produced a dose response curve which was very similar to sulphasalazine, incorporating the same stimulation of activity at low doses of 0.5 - 1.0 μM, followed by an inhibitory action at concentrations above 100μM. Furthermore, this activity appeared to be confined largely to the (-) optical enantiomer of gossypol (Table 1), which is thought to be responsible for the contraceptive properties of this compound (Waller et al, 1983; Matlin et al, 1985). At a concentration of 0.5μM, (-) gossypol significantly ($P<0.01$) stimulated 15-PGDH activity, from a control level of 7.17 ± 0.56 ng/mg/min to 9.71 ± 0.92, whilst at the same dose, (+) gossypol produced no significant effect. Neither enantiomer stimulated enzyme activity at a concentration of 1.0μM.

## D. Discussion.

These studies have demonstrated that the enzyme targeted by sulphasalazine in the large intestine, 15-PGDH, is also present in the testis and epididymis of both the rat and human. In both species, the activity of this enzyme increases in the distal portions of the epididymis paralleling, in the rat at least, the distribution of $PGE_2$, which increases in concentration by a factor of four during transit from the caput to the cauda epididymis (Gerozissis and Dray, 1977).

The exact biological role of the $PGE_2$ detected in the epididymis remains unknown. However, PGs have been shown to affect the movement of spermatozoa from both the rabbit (Schlegel et al, 1983) and the human (Schlegel et al, 1981), and are known to stimulate the fertilizing potential of human spermatozoa in a trans-species in vitro fertilization system (Aitken and Kelly, 1985). Sperm motility has also been correlated with seminal PGE levels (Isidori et al, 1980; Cosentino et al, 1984b).

The biochemical mechanisms by which PGEs influence sperm motility and fertilizing potential is also unclear. However, these agents are known to increase the cAMP content of spermatozoa (Heindel et al, 1978; Cosentino et al, 1982; Kelly et al, 1984; Aitken et al, 1986), an action which has been found to stimulate both the motility and fertilizing capacity of mature spermatozoa (see chapter 1). Such an increase in cAMP content may be achieved either directly by activation of the adenylate cyclase (O'Donnel, 1974), or indirectly via the induction of a calcium influx, through the ionophore-like activity of these compounds (Kirtland and Baum, 1972), which consequently causes an increase in cAMP levels (Aitken et al, 1986).

The influence of sulphasalazine on ulcerative colitis appears to involve the inhibition of PG catabolism, thus invoking the cytoprotective effects of the PGs which subsequently accumulate in the intestine (Hoult and Moore, 1980). However, the suppression of rat epididymal 15-PGDH by high doses (>100μM) of sulphasalazine is probably irrelevant in the context of contraception, as although such levels may be achieved in the bowel, it is

extremely doubtful whether such concentrations would be attainable in the peripheral circulation. Of greater interest in terms of male fertility control is the significant increase in 15-PGDH activity observed at sulphasalazine concentrations of around 0.5 - 1.0 μM. Intriguingly, similar concentrations of the male contraceptive agent gossypol also induced a corresponding increase in the activity of this prostaglandin catabolizing enzyme. Furthermore, when the resolved enantiomers of gossypol were examined for their influence on 15-PGDH activity, only (-) - gossypol was found to possess stimulatory activity. This is of significance because it is only the (-) enantiomer which is thought to be capable of disrupting fertility (Waller et al, 1983; Matlin et al, 1985).

Stimulation of 15-PGDH activity in vivo would be expected to lower the PG content of the epididymis. Some evidence that a reduction of PG levels within the male reproductive tract might disrupt fertility has been obtained from studies in which asprin (Biswas et al, 1978; Ratnasooriya and Lionel, 1984) and fenclozic acid (Ratnasooriya and Wadsworth, 1979) have been shown to exert a contraceptive action, presumably through the ability of these reagents to disrupt PG synthetase activity. Furthermore, the addition of exogenous 15-PGDH to the seminal plasma from rabbits (Schlegel et al, 1983) and man (Schlegel et al, 1981) has been shown to reduce sperm motility.

However, direct evidence for a lowering of testicular or epididymal prostaglandin levels following treatment with sulphasalazine or gossypol is, at present, lacking. Analysis of the seminal prostaglandin levels in patients receiving sulphasalazine treatment did not reveal any significant deviations from those of normal fertile males (Cosentino et al, 1984a). However, the prostaglandin content of seminal plasma is largely a reflection of the synthetic capacity of the seminal vesicles (Kelly, 1981), and is unlikely to reflect the levels obtained in the testis and epididymis.

Sulphasalazine undergoes azo-reduction in the intestine to produce 5-amino salicylic acid and sulphapyridine (Das et al, 1973a, b). The limited studies that have been carried out on the rat have shown that

sulphapyridine alone is capable of reducing fertility in this species (O'Morain et al, 1982; Pholpramool and Srikhao, 1983). Furthermore, 5-aminosalicylate has no effect on the fertility of either rats (O'Morain et al, 1982; Pholpramool and Srikhao, 1983) or humans (Cann and Holdsworth, 1984; Shaffer et al, 1984). However, although the putative contraceptive component of sulphasalzine, sulphapyridine, is capable of reducing the fertility of rats (O'Morain et al, 1982; Pholpramool and Srikhao, 1983), it is not as efficient in this respect as an equivalent dose of sulphasalazine. This is difficult to equate with the claim that sulphapyridine is the active metabolite, unless the bioavailabilty of the latter is a problem in the rat. These results may indicate that the parent compound itself plays an active role in the induction of temporary infertility. Whether sulphapyridine causes infertility in humans is unknown. If this compound was found to be active in man, it would obviously counteract the suggestion that the stimulation of 15-PGDH activity is a significant factor in the contraceptive action of either sulphasalazine or gossypol, since the in vitro studies described here indicate that sulphapyridine does not exert a significant effect upon this enzyme. In addition, the observation that 5-amino-salicylic acid stimulates 15-PGDH activity, whilst being ineffective in inducing infertility in man and rats (O'Morain et al, 1982; Pholpramool and Srikhao, 1983; Cann and Holdsworth, 1984; Shaffer et al, 1984) would also suggest that stimulation of 15 PGDH activity is not involved in the induction of infertility.

Analysis of the literature reveals reports of other sulphonamide type compounds causing infertility. Co-trimoxazole (a sulphonamide containing mixture) (Murdia et al, 1978), and dapsone (a sulphonamide related molecule) (Grieve, 1979) have both been found to reduce sperm numbers and sperm motility. A subsequent study has confirmed and extended these findings (Wong et al, 1987). Although the sulphonamide, sulphamethizole, did not cause infertility in rats dosed orally with this compound (Levi et al, 1982), this may give a clue as to the pharmacological basis of sulphasalazine's action, as sulphamethizole differs from sulphapyridine, the putative anti-fertility portion of sulphasalazine, in that it has no pyridine ring

in its structure. As sulphasalazine is known to inhibit both intestinal folate transport and the enzymes dihydrofolate reductase, methylene-tetrahydrofolate reductase and serine transhydroxymethylase (Franklin and Rosenberg, 1973; Selhub et al, 1978), it is possible that this compounds anti-fertility action may be mediated via effects on this vitamin. However the demonstration that patients receiving sulphasalazine therapy showed no improvement in their semen profile upon administration of large doses of folate (O'Morain et al, 1984), and that seminal folate levels in sulphasalazine treated patients were normal (O'Morain et al, 1985) suggest that these compounds are not involved in the antifertility action of sulphasalazine.

Recent reports that sulphasalazine affects the production of reactive oxygen species (Myachi et al, 1987), and that sperm membrane lipid peroxidation increases after sulphasalazine treatment (Chodorge et al, 1986) are of interest in view of the inverse relationship shown to exist between high levels of free oxygen radical production by human spermatozoa and their ability to fuse with and penetrate zona-free hamster eggs (Aitken and Clarkson, 1987). Although it has been stated that sulphasalazine itself does not constitute an acceptable male contraceptive (Giwercman and Skakkebæk, 1986) its action may provide clues for the development of a target-specific, non-hormonal male contraceptive.

In conclusion, these studies have demonstrated that the first enzyme of prostaglandin metabolism, 15-PGDH, is present in the testis and epididymis of both the rat and the human. The concentration of this enzyme was found to be greater in the corpus and caudal regions of the epididymis, and its activity could be modulated by the male contraceptive agents sulphasalzine and (-) gossypol. Available data suggests an important role for prostaglandins in the control of male reproductive function, and that agents which disrupt the synthesis or catabolism of this group of compounds may have potential as male contraceptive agents. Whether these particular contraceptive agents function through the disruption of PG metabolism is, however, debatable.

# Chapter 8. Investigation of the mechanism of action of propranolol, and its possible synergism with nonoxynol-9

## A. Introduction.

Spermicides exert an antifertility effect upon spermatozoa as these cells pass through the female genital tract. Currently, the nonionic detergent nonoxynol-9 (nonylphenoxy-polyethyleneoxyethanol) is the spermicide in widespread use, being used on the spermicidal condom, in spermicidal foam, creams, gels, suppositories and sponges. The basis of this compound's spermicidal activity resides in its ability to solubilise the sperm plasma membrane, causing rapid immobilization and cell death (Schill and Wolf, 1981; Wilborn et al, 1983).

However, as nonoxynol-9 is a surfactant, it may have problems contacting spermatozoa, particularly when they are entrapped in the coagulum formed after ejaculation, or if they rapidly enter the cervical mucus (Sharman et al, 1986; Zanaveld et al, 1986). Thus, several other compounds have been investigated as potential vaginal contraceptives. These include the widely used beta-blocking drug, propranolol, which is known to inhibit human sperm motility (Peterson and Freund, 1973, 1975; De Turner et al, 1978; Zipper et al, 1982).

The basis of this compound's spermicidal action is thought to depend upon its local anaesthetic properties, rather than its beta-blocking ability, as the purified D-isomer has been found to be as effective as the racemic mixture in supressing sperm motility, despite the fact that D-propranolol does not possess the ability to block beta-receptors (Peterson and Freund, 1973, Hong et al, 1981). Propranolol thus constitutes a potential candidate for development as a novel spermicidal agent (Chijioke et al, 1986). The possibility also exists that propranolol may interact synergistically with nonoxynol-9 to produce a novel potent "cocktail" spermicide. At the request of Family Health International, the sponsors of this Ph.D. fellowship, a study was conducted into both the mode of action of propranolol and its interaction with nonoxynol-9.

## B. Materials and Methods.

i. Sperm preparation. Semen samples were produced by masturbation into sterile plastic containers by donors exhibiting normal semen profiles (>20 x $10^6$ spermatozoa/ml; >40% progressive motility and >40% normal morphology - Aitken et al, 1982). The spermatozoa were separated from the seminal plasma by 3 cycles of centrifugation at 500g for 5 min in 8 ml volumes of medium BWW (a modified Krebs-Ringer bicarbonate buffer described by Biggers et al, 1971), and were finally resuspended at a sperm concentration of 20 x $10^6$ spermatozoa/ml and kept at 37°C prior to use.

ii. Motility analysis. The concentration of motile cells in washed sperm preparations was assessed by counting at least 100 cells in a 10µl aliquot at x250, with the aid of a grid on an eye-piece graticule. The effects of drugs on sperm motility in unwashed semen was determined using the Sander-Cramer test (Sander and Cramer, 1941), whereby 200µl of freshly ejaculated semen is mixed with 500µl of drug solution, and the percentage of motile spermatozoa present 20 secs after drug addition is estimated.

iii. Sperm Function. The assessment of human sperm function was carried out using the zona-free hamster oocyte assay introduced by Yanagimachi et al (1976). The acrosome reaction was induced by pre-incubating spermatozoa in the presence of the divalent cation ionophore A23187, prepared as an aqueous suspension of the $Ca^{2+}/Mg^{2+}$ salt (0.026mg/ml) (Aitken et al, 1984). Capacitated spermatozoa were then washed free of A23187 at the end of the pre-incubation phase and subsequently incubated with zona-free hamster oocytes for 3 h in fresh BWW. In order to assess the incidence of sperm-oocyte fusion, the oocytes were washed to remove loosely adherent spermatozoa and then compressed to a depth of about 30µM before examination at x250 by phase

contrast microscopy. The percentage of oocytes containing decondensed sperm heads with an attached or closely associated tail, as well as the mean number of penetrations per oocyte, were recorded. The number of oocytes incorporated into each assay (20-30) was sufficient to ensure an adequate sperm-oocyte collision frequency (Aitken and Elton, 1986). In addition, corrections for small differences in the concentration of motile spermatozoa were made using the formula, based on Poisson distribution theory, to adjust all results for a constant concentration of motile spermatozoa (5 x $10^6$/ml), where $P_2=1 - \exp( - rm_1)$, in which $P_2$ = the corrected penetration rate for a motile sperm concentration of 5 x $10^6$/ml, r = the ratio of the theoretical concentration of motile spermatozoa (ie 5) to the actual concentration recorded in the experiment, and $m_1$ = the observed outcome of the pernetration test in terms of the mean number of spermatozoa penetrating each oocyte (Aitken and Elton, 1984).

iv. Intracellular calcium measurements. The influence of propranolol on the intracellular concentration of free, extramitochondrial $Ca^{2+}$ was assessed by monitoring the fluorescence signal emitted by the calcium chelating agent, Quin-2, originally described by Tsien et al (1982), following the methodology outlined by Irvine and Aitken (1986). Briefly, spermatozoa were washed 3 times with medium BWW, and finally resuspended at a total sperm concentration of 20 x $10^6$/ml. Quin-2-tetracetoxymethyl ester was added from a stock solution in dimethylsulphoxide to achieve a final concentration of 50μM, and the cell suspension was then incubated at 37°C in 5% $CO_2$ in air for 20 min. The cell suspension was then diluted 1:9 with fresh BWW and the incubation continued for a further 100 min. The cells were subsequently washed twice by centrifugation at 500g to remove extracellular Quin-2, and finally resuspended in fresh medium BWW to a concentration of 10 x $10^6$/ml. Fluorescence measurements were performed in a Perkin-Elmer spectrofluorimeter, with the excitation wavelength set at 339nm and the emission wavelength at 492 nm. The suspension of

spermatozoa was maintained at 37°C in quartz cuvettes for the duration of each series of measurements. The entry of Quin-2 into the cells was checked by observing the shift in the fluoresence maximum from 430 to 492nm. The intracellular calcium content was calculated using the equation $[Ca^{2+}]_i = K_d(F-F_{min})/(F_{max}-F)$, where $K_d$ = 115nM (Tsein et al, 1982). $F_{max}$ and $F_{min}$ were determined by the addition of 0.5% v/v triton X-100, followed by 10mM EGTA.

v. Reagents. Purified D-propranolol was provided by Family Health International (Research Triangle, North Carolina, USA). All other biochemicals were obtained from either Sigma (Poole, England), or BDH (Glasgow, Scotland) and were of the highest purity grade available.

vi. Statistics. All statistical comparisons were made using the non -parametric Mann-Whitney U test.

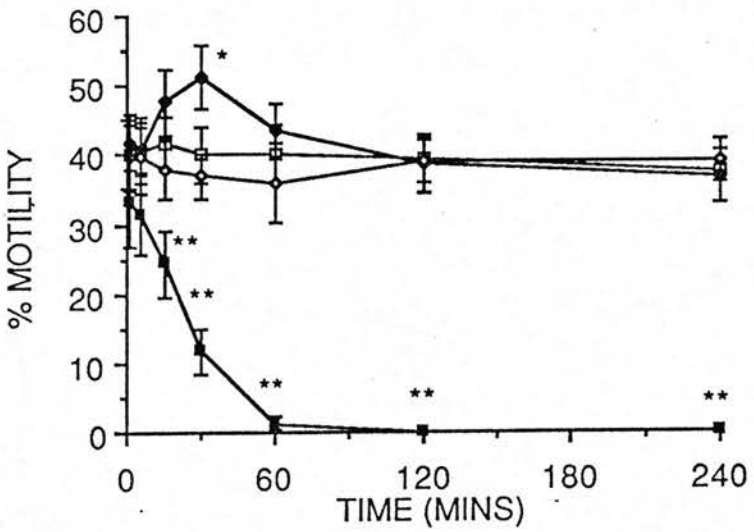



Figure 54. Percentage motility expressed by human spermatozoa incubated in either medium BWW alone (open squares), or medium BWW plus DL propranolol at a concentration of 5 (closed diamonds), 50 (open diamonds), or 500 (closed squares) μM. Values shown are the mean ± sem of six separate determinations. Significance levels are; *, $P<0.05$, **, $P<0.01$.

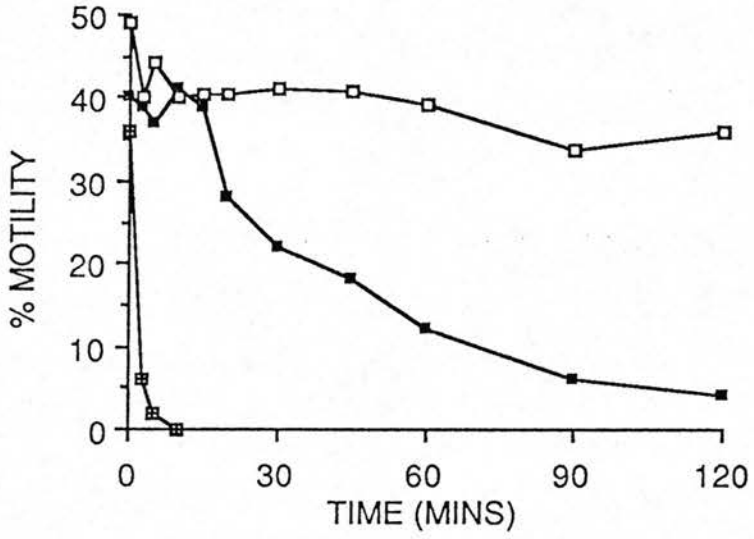



Figure 55. Percentage motility expressed by two different human sperm samples (hatched and closed squares) incubated in medium BWW plus DL propranolol at a concentration of 500 μM. Control values (open squares) are the mean of six separate determinations performed in medium BWW alone.

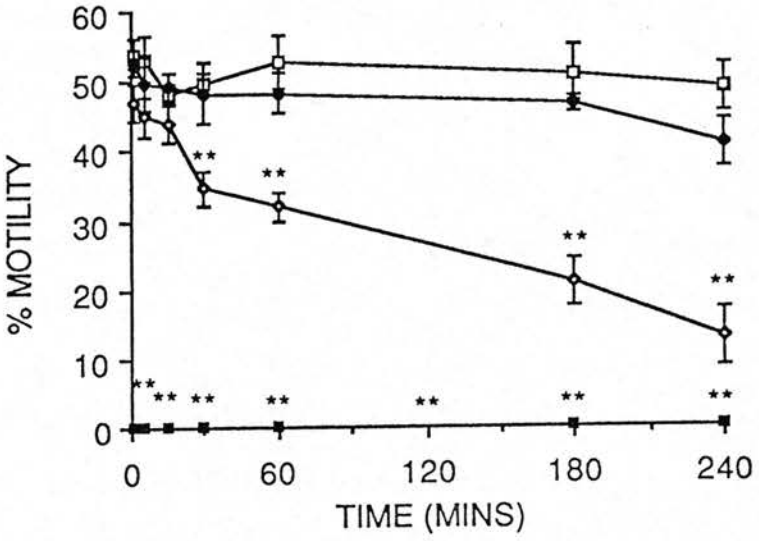



Figure 56. Percentage motility expressed by human spermatozoa incubated in either medium BWW alone (open squares), or medium BWW plus nonoxynol-9 at a concentration of 5 (closed diamonds), 50 (open diamonds), or 500 (closed squares) µg/ml. Values shown are the mean ± sem of six separate determinations. Significance levels are; **, $P<0.01$.

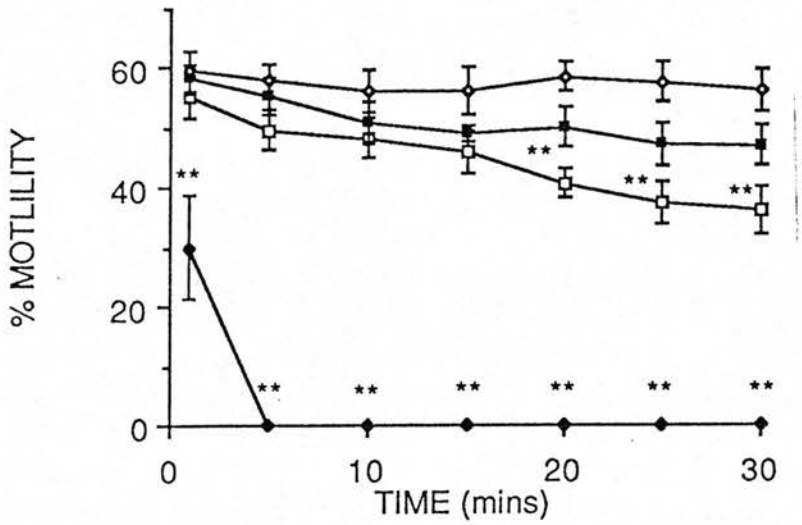



Figure 57. Percentage motility expressed by human spermatozoa incubated in either medium BWW alone (open diamonds), or medium BWW plus 75µg/ml nonoxynol-9 (open squares), 500µM DL propranolol (closed squares), or both of these reagents at the doses already stated (closed diamonds). Values shown are the mean ± sem of six separate determination. Significance levels are; ** $P<0.01$.

## C. Results.

i. Motility analysis. Time and dose dependant studies utilising the racemic mixture of propranolol revealed that, when added at the relatively low dose of 5μM, this reagent caused a transient stimulation of human sperm motility, 30 mins after addition. When present at a concentration of 50μM, this compound had no significant effects on sperm movement. However, at the higher concentration of 500μM, DL propranolol caused a significant ($P<0.01$) suppression of motility within 15 mins of addition, and after 1 h of incubation, motility had been reduced to negligible levels (Fig 54). However, the response of different semen samples to this compound varied greatly, as can be seen in Fig 55, with some samples showing complete loss of motility within 10 min of the addition of 500μM DL propranolol, whereas other samples continued to show low levels of motility as long as 2 hours after the addition of this concentration of propranolol.

Time and dose dependant analysis of the effects of nonoxynol-9 revealed that at a concentration of 5μg/ml, this reagent had no significant effect on human sperm motility over a period of 240 mins. However, at a dose of 50 μg/ml, this drug caused a significant ($P<0.01$) reduction of motility within 30mins of addition. At a concentration of 500μg/ml, nonoxynol-9 completely abolished all sperm movement within 1 min of addition (Fig56).

To investigate the interaction of these two spermicidal agents, a concentration of each compound was chosen which caused only a gradual inhibition of sperm motility. When incubated in the presence of DL-propranalol at a concentration of 500μM, no significant effect upon motility was seen after 30 mins of incubation. Nonoxynol-9 at a dose of 75μg/ml caused a slight but significant ($P<0.01$) reduction in motility by 20 mins after the addition of this reagent. However, when both compounds at these concentrations were added to incubations of human spermatozoa, motility was reduced by 50% within 1 min of addition, and was completely abolished after 5 mins of incubation (Fig 57).

Dose and time dependant studies of the possible spermicidal effects

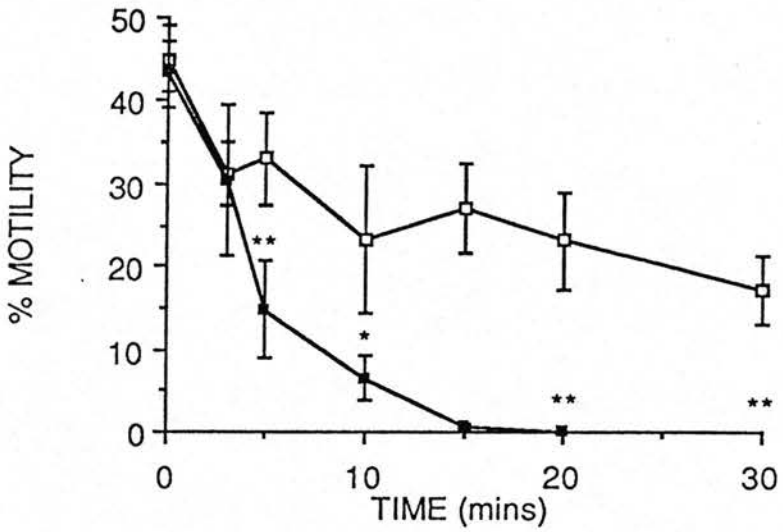



Figure 58. Percentage motility expressed by human spermatozoa incubated in the presence of 500μM DL propranolol in either medium BWW (open squares), or calcium free BWW (closed squares). Values shown are the mean ± sem of six separate determination. Significance levels are; *, $P<0.01$; **, $P<0.01$.

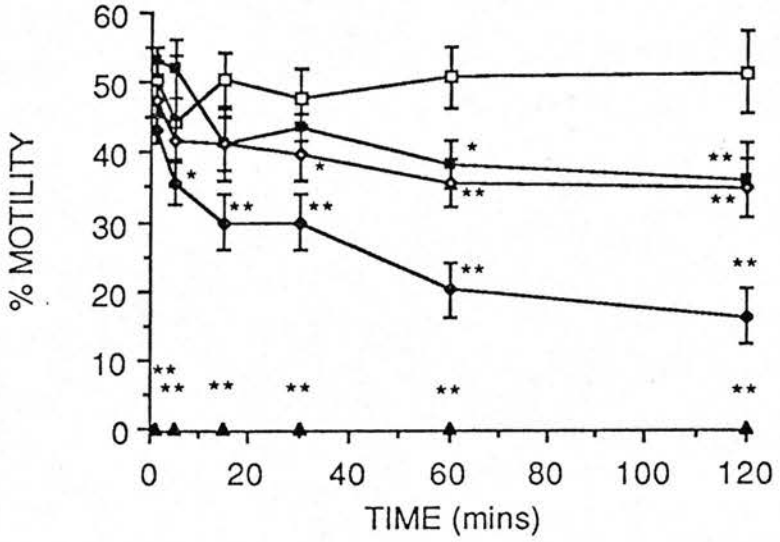



Figure 59. Percentage motility expressed by human spermatozoa incubated in either medium BWW alone (open squares), or medium BWW plus D propranolol at a concentration of 5 (closed squares), 50 (open diamonds), or 500 (closed squares), or 5000 (closed triangles) μM. Values shown are the mean ± sem of six separate determinations. Significance levels are; *, $P<0.05$, **, $P<0.01$.

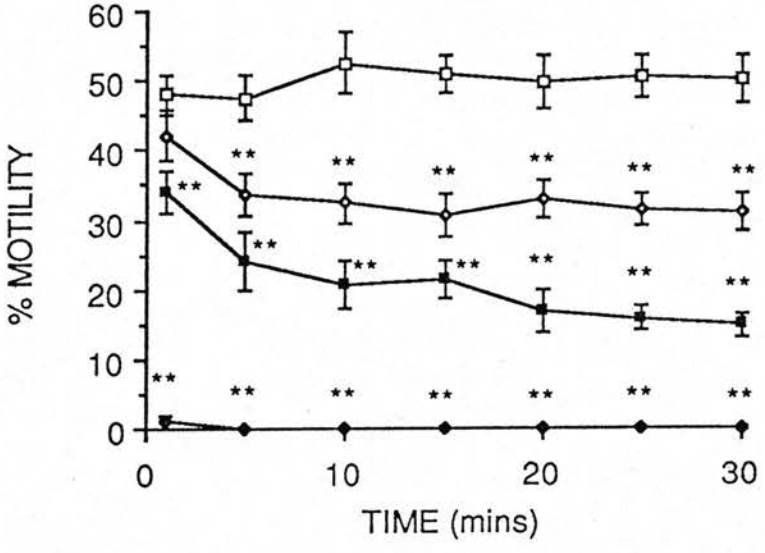



Figure 60. Percentage motility expressed by human spermatozoa incubated in either medium BWW alone (open squares), or medium BWW plus 75µg/ml nonoxynol-9 (closed squares), 500µM D propranolol (open diamonds), or both of these reagents at the doses already stated (closed diamonds). Values shown are the mean ± sem of six separate determination. Significance levels are; **, P<0.01.

of purified D propranolol were also investigated. As with racemic propranolol, this resolved enantiomer caused a transient stimulation of sperm motility soon after addition at a concentration of 5μM, although this increase was not statistically significant. However, in contrast to the effects of the unresolved form of this drug, D propranolol at a concentration of 5μM caused a significant ($P<0.01$) reduction in percentage motility over an incubation period of 120 mins. A similar response profile to that expressed in the presence of 5μM D propranolol was found for 50μM of this reagent. In contrast to the effects of DL propranolol, when added at a concentration of 500μM, D propranolol did not completely abolish sperm movement over a 120 min incubation period, although a significant ($P<0.01$) suppression of sperm motility did occur. When added at a concentration of 5mM, D propranolol caused a total inhibition of sperm movement within 1 min of addition (Fig 59).

In view of the apparent synergism between DL propranolol and nonoxynol 9 in the suppression of sperm movement, a study was undertaken of the interaction between nonoxynol-9 and D propranolol in the disruption of sperm motility. Incubation in the presence of nonoxynol-9 at a dose of 75μg/ml caused a slight but significant ($P<0.01$) suppression of sperm movement. D propranolol at a concentration of 500μM caused a significant ($P<0.01$) reduction in motility by 1 min after its addition. However, when both these compounds at these concentrations were added to incubations of human sperm, motility was almost completely abolished within 1 min of addition, and was reduced to zero after 5 mins of incubation (Fig 60).

To determine whether these drugs would interact positively to inhibit sperm motility in a spermicidal context, their effects both alone and in combination upon the movement of spermatozoa in unwashed semen were determined. For this study, concentrations of the individual reagents were chosen which had only a slight effect on sperm motility on their own. When added at a concentration of 300 μg/ml, nonoxynol-9 had a slight, but insignificant suppressive effect on seminal sperm motility within 20 secs of

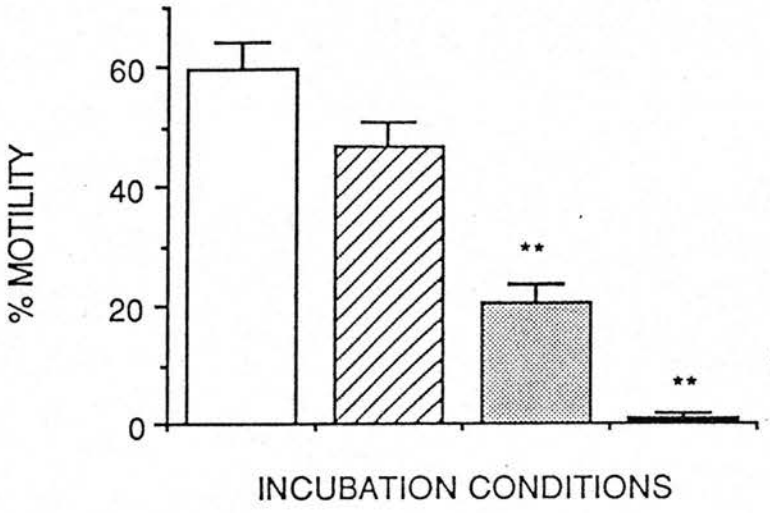



Figure 61. Percentage motility expressed by unwashed human spermatozoa incubated for 20 secs in the presence of vehicle alone (medium BWW - open bar), 300μg/ml nonoxynol-9 (lined bar), 850μM D propranolol (dotted bar), or both of these reagents at the doses already stated (closed bars). Values shown are the mean ± sem of six separate determination. Significance levels are; **, $P<0.01$.

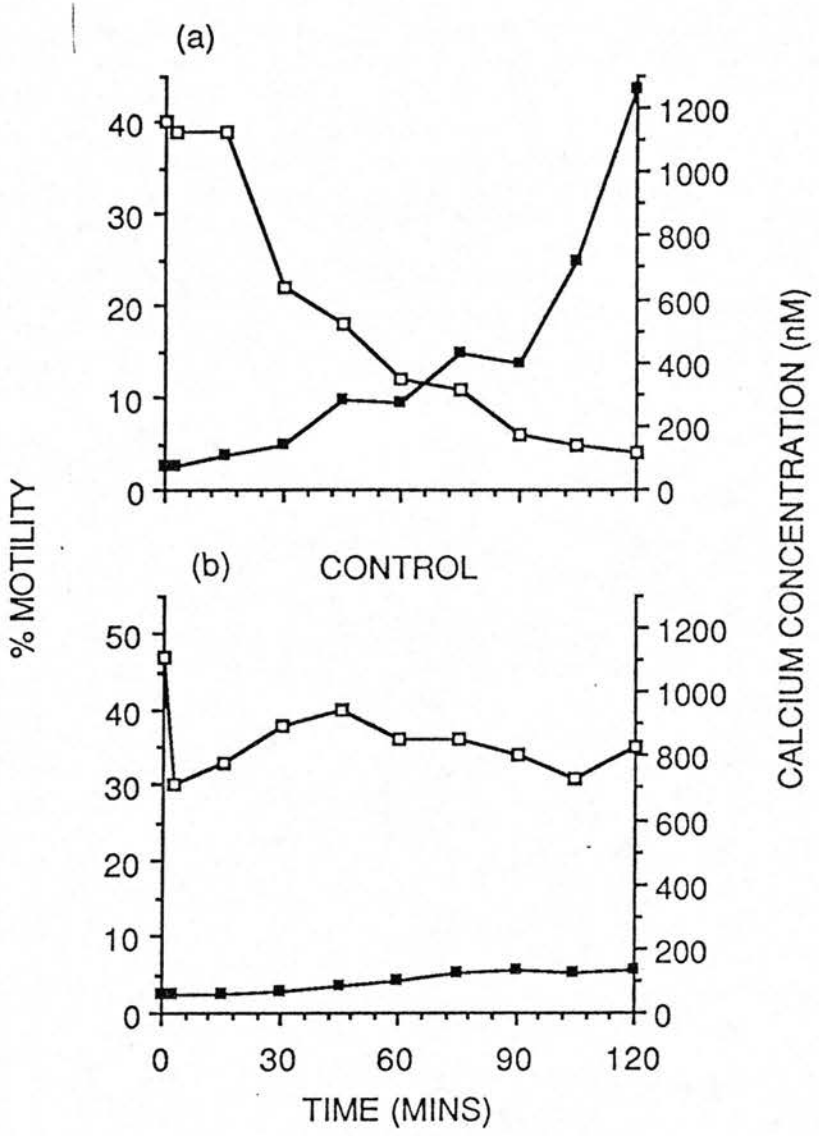



Figure 62. Free intracellular calcium content (closed squares) and percentage motility (open squares) determined for human spermatozoa incubated in either medium BWW alone (b), or in medium BWW plus 500μM DL propranolol (a).

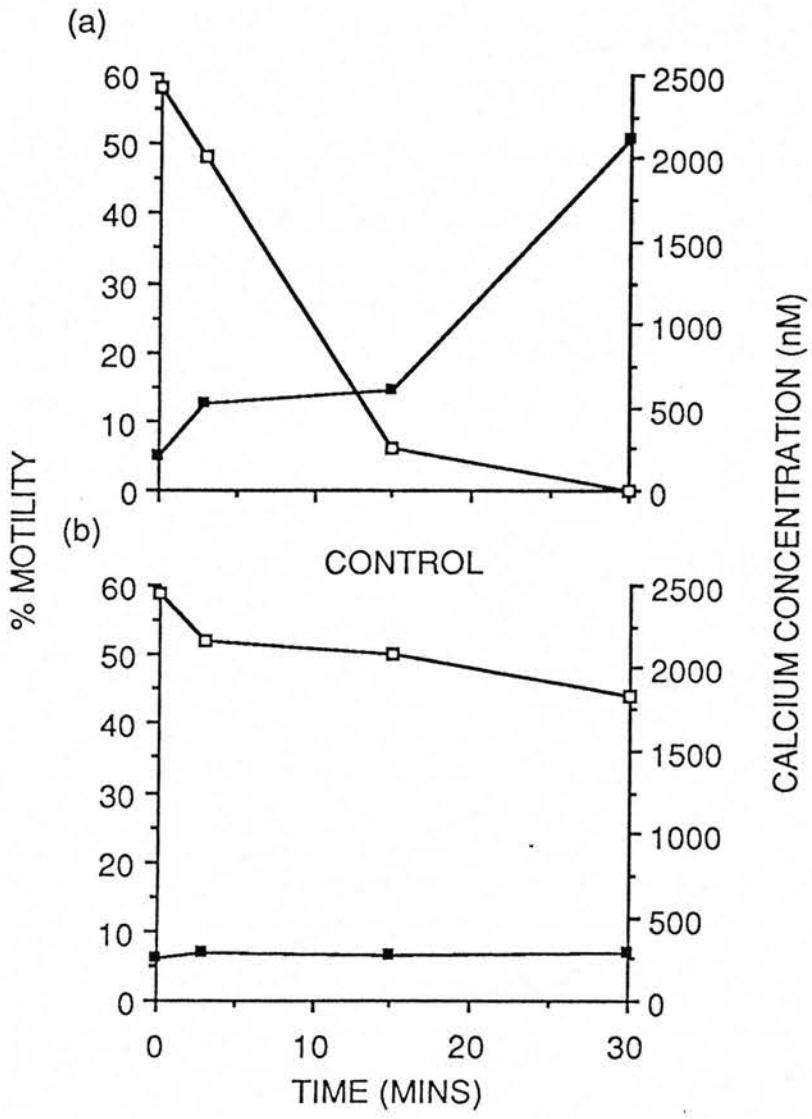



Figure 63. Free intracellular calcium content (closed squares) and percentage motility (open squares) determined for human spermatozoa incubated in either medium BWW alone (b), or in medium BWW plus 500μM DL propranolol (a).

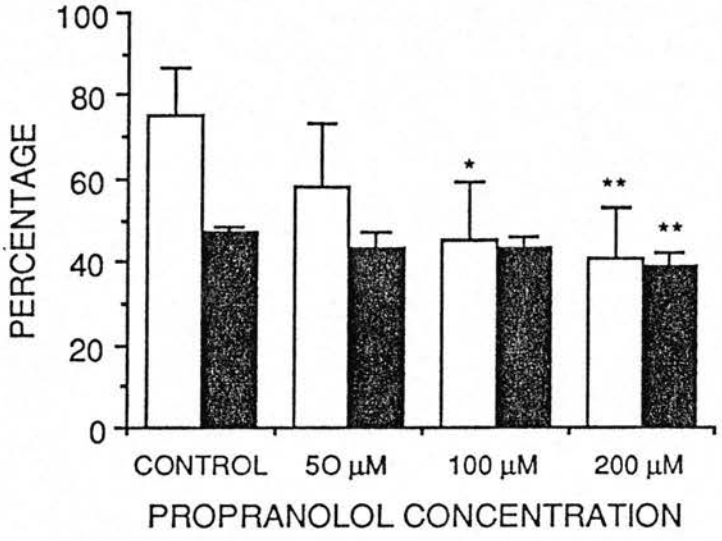



Figure 64. Corrected percentage penetration of zona-free hamster oocytes (open bars) and percentage motility expressed by human spermatozoa inubated in the presence of 0, 50, 100 and 200µM DL propranolol. Values shown are the mean ± sem of six separate determination. Significance levels are; *, $P<0.05$; **, $P<0.01$.

drug addition. D propranolol at a dose of 850μM significantly ($P<0.01$) inhibited sperm movement within 20 secs, although approximately 20% of cells were still motile after this time. However, when both drugs were added at these concentrations, motility was reduced almost to zero within this short incubation period, and those which exhibited movement at this time showed only feeble flagellar twitching (Fig 61).

ii. Intracellular calcium measurements. To investigate the mechanism of action of DL propranolol, intracellular calcium levels were analysed using the fluorescent probe quin-2. These measurements revealed that the suppression of motility caused by a concentration of 500μM of this reagent was associated with a concomitant increase in the free calcium content of these spermatozoa (Figs 62, 63).

To determine whether this calcium influx was a causative factor in the induction of motility loss, experiments were conducted in which DL propranolol was added to spermatozoa in the absence of exogenous calcium. The suppression of motility resulting from treatment with 500μM DL propranolol was found to be significantly ($P<0.01$) enhanced by incubation in calcium free medium BWW, with percentage motility in this medium being reduced to zero within 20 mins of addition of propranolol (Fig 58).

iii. Analysis of sperm function. To determine whether low doses of propranolol affected some aspect of sperm function other than motility, the ability of spermatozoa to fuse with zona-free hamster oocytes in the presence of different concentrations of DL propranolol was measured. These experiments revealed that at a dose of 100μM, this reagent significantly ($P<0.05$) inhibited the ability to penetrate hamster oocytes, whilst having no significant inhibitory affect on sperm motility. Although 200μM caused a significant ($P<0.01$)inhibition of sperm-oocyte fusion, this concentration of DL propranolol also significantly ($P<0.01$) suppressed sperm movement. (Fig 64). However, it should be noted that the reduction of motility seen at this dose of DL propranolol was not the cause of the

reduction in sperm-oocyte fusion, since the results presented in Fig 64 refer to a fixed concentration of $5x10^6$ motile cells per ml at all doses examined.

## 4. Discussion.

Although a wide variety of vaginal contraceptives are currently in use, all utilize the non-ionic surfactant nonoxynol-9 as their active ingredient. This compound acts by disrupting the sperm plasma membrane, causing rapid immobilization and cell death (Schill and Wolf, 1981; Wilborn et al, 1983). However, the relatively short period of protection provided by nonoxynol-9 (John Hopkins University Population Report, 1984), and fears over the possible teratogenic side-effects of this compound (Louis and Pearson, 1985) have stimulated the search for alternative spermicidal agents. In addition to producing more effective vaginal contraceptives, the development of alternatives to nonoxynol as a spermicidal agent is also of importance for developing countries, where the importing of raw materials makes the cost of widespread spermicide production prohibitively expensive. In China, for instance, attempts are being made to utilize a by-product of the petroleum industry, agent 741[alkylphenoxy polyethoxy ethanol (10)] as novel spermicidal agent (Xiao-Hui et al, 1986).

This study has confirmed the findings of other groups by showing that the beta-blocking drug, propranolol, inhibits human sperm motility (Peterson and Freund, 1973, 1975; De Turner et al, 1978; Zipper et al, 1982). In addition, the purified D enantiomer was also found to suppress sperm motility as has been previously reported (Peterson and Freund, 1973; Hong et al, 1981). This observation implies that the basis of propranolol's spermicidal action depends more upon its local anaesthetic properties, rather than its beta-blocking potential, as D-propranolol does not possess the ability to block beta-receptors (Barrett and Cullum, 1968). However, these experiments have also shown racemic propranolol to be more effective in supressing sperm motility than the purified dextro-isomer. Incubations performed in the presence of 500μM DL propranolol rendered spermatozoa completely immotile within 120 mins of addition of this reagent. Treatment with the same concentration of D propranolol did induce a significant ($P<0.01$) reduction in the percentage of spermatozoa exhibiting

flagellar movement, but after 120 mins of incubation, 17% of spermatozoa were still motile, significantly ($P<0.01$) greater than in the presence of the racemic mixture. Caution must be exercised in the interpretation of this discrepancy, however, as a significant ($P<0.01$) variation in response to propranolol was found to exist between different sperm samples. Furthermore, at the lower concentrations of 5 and 50μM, D propranolol caused a significant ($P<0.01$) suppression of sperm motility, whilst at these doses, the racemic mixture had no such inhibitory effects. Thus, the difference between the effects of DL and D propranolol on sperm motility may be due to the variation between different sperm samples, rather than to the differing pharmacological properties of these compounds.

Spermatozoa incubated in the presence of 50μg/ml nonoxynol-9 gradually lost the capacity for movement over a period of 60 mins, whereas at a concentration of 500μg/ml, this reagent caused immediate and complete loss of motility. To discern whether the anti-motility effects of propranolol and nonoxynol-9 could interact in a synergistic manner, spermatozoa were incubated in the presence of concentrations of these compounds which separately had only slightly suppressive effects on sperm motility. When added together, these compounds caused an immediate suppression of motility, with sperm movement being inhibited completely within five minutes of incubation. The combination of D propranolol and nonoxynol-9 also appeared to suppress sperm movement to a greater extent than would be expected if their effects were purely additive. These results thus show that propranolol and nonoxynol-9 can interact synergistically in their ability to inhibit sperm motility. Furthermore, this capacity was also expressed in a more realistic contraceptive setting, when these compounds were presented to spermatozoa still resident in seminal plasma. The basis for this synergism is unknown. However, this capacity probably reflects the fact that these compounds are affecting different aspects of membrane function. Possibly propranolol's stabilising effects are causing the sperm membranes to become more readily affected by the detergent properties of nonoxynol-9.

Measurement of the fluoresence signals emitted by human

spermatozoa loaded with the calcium probe Quin-2 revealed that an increase in free intracellular calcium content accompanied the inhibition of motility which resulted from treatment with DL propranolol. In view of the fact that high levels of calcium inhibit sperm motility (see chapter 5; Breitbart et al, 1985), and that the ionophores A23187 and nigericin are more potent than other membrane active agents in inducing immotility in human spermatozoa (Hong et al, 1985, 1986), the possibility exists that the alteration of membrane structure following treatment with membrane-stabilising reagents influences sperm motility by either stimulating calcium transport, or, more likely, inhibiting calcium efflux across the plasma membrane.

To investigate whether the inhibition of sperm motility which results from treatment with DL propranolol may be due to its ability to increase internal calcium levels, the effects of this reagent on sperm motility were tested both in the presence and the absence of extracellular calcium. The addition of 500μM DL propranolol to spermatozoa incubated in calcium free BWW (BWW with no added calcium and 100μM EGTA) caused the complete cessation of motility within 20 mins of addition, a significant ($P<0.01$) enhancement of the spermicidal action of this compound over that expressed in complete medium BWW. It would thus appear unlikely that the ability of DL propranolol to inhibit sperm motility is mediated by an increase in calcium levels. As the ability of DL propranolol to inhibit sperm movement was potentiated by the removal of calcium from the medium, and as the calcium concentration in BWW is probably far in excess of the levels of this cation that spermatozoa encounter upon ejaculation (Mann, 1983), propranolol may prove to be a more efficient spermicidal agent in vivo than in vitro. It is unclear why propranolol's efficiency in suppressing sperm movement should be increased in the absence of exogenous calcium calcium. However, the exisitence of such an interaction raises the possibility of utilizing combinations of propranolol and chelating agents as novel and effective spermicidal agents.

Analysis of the effects of low doses of DL propranolol show that this

agent also caused a significant inhibition of the penetration of zona-free hamster eggs when present at concentrations which produced no visible suppression of motility. This effect also probably reflects an action at the level of the sperm membrane, reducing the capacity for membrane fusion as a result of the membrane stabilising properties of propranolol. Such an ability further increases the contraceptive potential of propranolol.

Recently, considerable interest has been expressed over the anti-aids potential of nonoxynol-9. The capacity of this chemical to inactivate HTLV-III (Hicks et al, 1985; Voeller, 1986) makes a strong case for the continued use of this compound in spermicidal preparations. Thus the development of a "cocktail" spermicide containing both propranolol with nonoxynol-9 is attractive, as such a combination would have improved spermicidal efficacy, whilst retaining the protection against possible viral infection. However, the consequent reduction of the concentration of nonoxynol-9 required for spermicidal purposes, due to the inclusion of propranolol, may enhance the acceptability of this product through the reduction of side-effects. As clinical trials of DL propranolol have revealed this agent to be both safe and effective as a vaginal contraceptive (Zipper et al, 1983), the way would seem open for the development of a new, "combination" spermicidal agent.

# Chapter 9. General Discussion.

There are only three options currently available to the male who wishes to regulate his fertility; coitus interruptus, condoms and vasectomy. None of these methods provide the male equivalent of the effective, convenient and reversible female pill. The development of a safe, reliable and reversible male contraceptive would thus be a major breakthrough, especially for those couples who wish to transfer the burden of contraception from the female to the male, possibly as a result of contra-indications against the use of the female pill.

The male reproductive process presents two separate areas open to manipulation in a contraceptive context. The first, the formation of spermatozoa in the testes, is a continuous and complex process of cell division and differentiation, regulated by pituitary gonadotropins and gonadal peptides (Dizerega and Sherins, 1980). The second involves the maturation of spermatozoa within the epididymis, the biochemical complexities of which are only now being fully comprehended (see part A). The passage of spermatozoa through the female tract, and ultimately the fertilization of the oocyte present further opportunities for the artificial inhibition of sperm function.

Attempts to inhibit spermatogenesis have been hampered by the almost inevitable interference with testicular steroid secretion that results from this approach to male contraception. To compensate for the loss of libido, potence and secondary sexual characteristics which results from the decrease in testosterone secretion, androgen substitution therapy is required. This, in turn, presents its own problems, as such treatment tends to counteract the original anti-spermatogenic effects. Furthermore, inhibition of spermatogenesis rarely induces complete azoospermia; more often only severe oligozoospermia is achieved. It would thus appear that the development of methods which interfere with the maturation of spermatozoa in the epididymis represents the most attractive approach to the development of novel forms of male contraception. A chemical attack centred upon spermatozoa maturing in the epididymis would minimise the risk of damage to the genetic material, as cell divisions have been

completed by this stage of development, and the DNA is in a highly condensed state. Furthermore, such a site of action would ensure a rapid onset of infertility, requiring a relatively short period of treatment before unprotected intercourse became safe.

α-chlorohydrin, 6-chloro-6-deoxysugars, gossypol and sulphasalazine all have an antifertility action in the male which appears to act either partially or completely at the level of the epididymis. However, In view of the toxicity of α-chlorohydrin (Jackson et al, 1977), the demonstration that the central nervous system of marmosets, mice and rats is adversely affected by 6-chloro-6-deoxysugars (Jacobs and Duchin, 1980; Jacobs and Ford, 1981; Ford and Waites, 1981; Ford, 1982b), the incomplete reversibility of the anti-spermatogenic effects of gossypol (Prasad and Diczfalusy, 1982), and the numerous side effects associated with sulphasalzine treatment, such as nausea, vomiting, headaches, fever, reticulocytosis, arthralgia, blood dyscrasia, bronchospasm, pulmonary eosinophilia and peripheral neuropathy (Toth, 1979), none of these compounds have been introduced as novel male contraceptive agents. However, all these reagents provide valuable clues, not only for the development of target specific, non-steroidal male contraceptives, but also for the identification of those lesions responsible for naturally occurring male infertility.

As over 40% of cases of male infertility have no demonstrable cause (Diczfalusy, 1986), research aimed at defining the causative mechanisms of such ailments may, in turn, reveal fundamental processes open to disruption in fertile spermatozoa. In this context, the correlation shown to exist between free oxygen radical production, lipid peroxidation and the ability to fuse with and penetrate zona-free hamster eggs (Aitken and Clarkson, 1987) is an exciting observation, especially in the light of the knowledge that sulphasalazine affects the production of reactive oxygen species (Myachi et al, 1987), and that sperm membrane lipid peroxidation increases after sulphasalazine treatment (Chodorge et al, 1986). Spermatozoa are highly

susceptible to damage by reactive oxygen species because of the high levels of unsaturated fatty acids, particularly docosahexanoic acid, present in this cell type (Jones et al, 1979), and because they are unable to protect themselves against peroxidative damage (Aitken and Clarkson, 1987). The observation that eight other sulphonamide compounds cause infertility in male rats (Wong et al, 1987) is of great interest. Further research is required to determine whether this class of substances also induce lipid peroxidation in spermatozoa, thus possibly creating a unifying hypothesis for the development of a new antifertility agent for the male.

The search for a biochemical male contraceptive agent continues. Novel anti-fertility agents are constantly being evaluated, many from plants used as traditional herbal medicines. Tripterigium wildfordii, a chinese herb medicine (Zheng et al, 1983, 1985; Qian et al, 1986a) and Allitridum, the active principle of garlic (Qian et al, 1986b) both possess antifertility activity, and as shown in chapter 8, propranolol may soon be utilized as a new spermicide, either on its own, or more probably in combination with nonoxynol-9. New approaches to contraception are also being adopted. ORF 13904, a long-chain, sulfonated polystyrene polymer, is not a surfactant, nor does it possess spermicidal properties, yet it exhibits potent vaginal contraceptive activity by inhibiting the penetration of cervical mucus (Homm et al, 1985), in addition to inhibiting acrosin activity (Foldesy et al, 1986). 1,2,3-trihydroxypropane has been found to cause long-term cessation of spermatogenesis upon injection into the rat testis, without apparently affecting leydig cell function, libido, secondary sexual characteristics, potency or serum hormone levels (Weibe and Barr, 1984). Certain 1-substituted imidazoles are effective spermicidal agents which can be administered orally (Eliasson and Dornbusch, 1980; Vickery et al, 1983). Compounds which prevent the spontaneous liquefaction of the coagulum formed by semen upon ejaculation may also have potential as novel vaginal contraceptives (Mandal and Bhattacharyya, 1986). Reducing the transit time for the passage of spermatozoa through the epididymis by treatment with sympathomimetic drugs (Hib, 1976; Ratnasooriya et al, 1980) may also

provide a new approach to fertility control in the male.

New analogues of contracepive agents previously discarded because of insufficient antifertility activity, or deleterious side-effects, are also being developed. For instance, the 1H-indazole-3-carboxylic acids, known to exert antispermatogenic activity (Corsi et al, 1976), were discarded as potential male contraceptive agents because of their nephrotoxicity in monkeys (Barcellona et al, 1986). However, interest has recently been rekindled with the testing of tolnidamine (1-[(4-chloro-2-methylphenyl)methyl]-1H-indazole-3-carboxylic acid), which apparently possesses the antispermatogenic effects, but not the toxicity, of its parent compound (Barcellona et al, 1986).

Although treatment with depot medroxyprogesterone acetate and testosterone enanthate was found to cause a severe suppression of sperm numbers (Alvarez-Sanchez et al, 1977; Brenner et al, 1977; Frick et al, 1977a, b; Melo and Coutinho, 1977; Sanchez et al, 1979; Bain et al, 1980), the inability of this combination of compounds to induce complete azoospermia caused this approach to male contraception to be temporarily abandoned. However, the recent demonstration that the spermatozoa which remain after such treatment are unable to fuse with zona-free hamster ova (Wu and Aitken, unpublished observations) has reinitiated interest in this area of male contraceptive research. Similar studies in progress using testosterone enanthate alone may reveal actions of this anti-fertility agent overlooked using conventional semen analysis (J Aitken, personal communication).

The demonstration that spermatozoa from sub-fertile donors express lower levels of hyperactivated motility (Burkman, 1984; Robertson et al, 1987) indicates that inhibition of this pattern of movement may provide a subtle approach to male contraception. In this context, the demonstration in this study of the relationship between cAMP and hyperactivated motility suggests that the inhibition of this nucleotide could present new means of suppressing sperm function. The adenylate cyclase in spermatozoa exhibits many unique characteristics, in that it does not respond to the usual stimulators of adenylate cyclase activity, such as fluoride, guanyl

nucleotides, cholera toxin or forskolin (Braun and Dods, 1975; Herman et al, 1976; Chen and Boettcher, 1979; Garbers and Kopf, 1980; Stengel et al, 1982; Forte et al, 1983). It is thought that this discrepancy is due to the lack of a stimulatory subunit in this cell type (Stengel and Hanoune, 1984; Hildebrandt et al, 1985). Due to the unique nature of the spermatozoal form of adenylate cyclase, this enzyme constitutes an attractive potential target for a new antifertility agent.

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